UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

DEPARTAMENTO DE NUTRICIÓN, BROMATOLOGÍA Y TECNOLOGÍA DE LOS ALIMENTOS

TESIS DOCTORAL

Tratamientos combinados de altas presiones y bioconservación en la mejora de la seguridad microbiológica de productos cárnicos listos para el consumo

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

María de Alba Ortega

Director

Margarita Medina Fernández-Regatillo
Daniel Bravo Vázquez

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TRATAMIENTOS COMBINADOS DE ALTAS PRESIONES Y BIOCONSERVACIÓN EN LA MEJORA DE LA SEGURIDAD MICROBIOLÓGICA DE PRODUCTOS CÁRNICOS LISTOS PARA EL CONSUMO

María de Alba Ortega

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Memoria presentada por **María de Alba Ortega** para la obtención del grado de Doctor por la Universidad Complutense de Madrid

Directores: Dr. Margarita Medina Fdez-Regatillo y Dr. Daniel Bravo Vázquez

Departamento de Tecnología de Alimentos, INIA

EL DOCTORANDO

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Margarita Medina Fdez-Regatillo, Investigadora A1, y Daniel Bravo Vázquez, Investigador Contratado, del Departamento de Tecnología de Alimentos del INIA,

CERTIFICAN:

Que la tesis titulada "Tratamientos combinados de altas presiones y bioconservación en la mejora de la seguridad microbiológica de productos cárnicos listos para el consumo" de la que es autora María de Alba Ortega, ha sido realizada bajo su dirección en el Departamento de Tecnología de Alimentos del INIA, y cumple las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid.

Madrid, 12 de Diciembre de 2013

Fdo. Margarita Medina

Fdo. Daniel Bravo

A mi familia , en especial a mi abuelo José

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DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA III
HIGIENE Y TECNOLOGÍA DE LOS ALIMENTOS
FACULTAD DE VETERINARIA
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RESUMEN de TESIS

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Las tendencias actuales del consumidor hacia alimentos menos tratados, nutritivos, más estables y seguros, con una vida útil más larga y más fáciles de preparar, ha aumentado la demanda de productos listos para el consumo (o RTE, del inglés Ready-To-Eat). Estos alimentos se consumen directamente sin tratamiento higienizante o con un procesado mínimo y pueden contaminarse con microorganismos patógenos durante las operaciones de manipulación y procesado. *Listeria monocytogenes*, varios serovares de *Salmonella* spp. y *Escherichia coli* (incluido O157:H7) pueden alcanzar estos productos durante su preparación, aumentando el riesgo para el consumidor si estos productos se almacenan en condiciones de abuso moderado o aumento incontrolado de temperatura.

En el presente trabajo, se propone la aplicación de las altas presiones hidrostáticas (APH), al ser una tecnología de conservación no térmica encaminada a aumentar la seguridad microbiológica y la vida útil de los alimentos, en combinación con distintas estrategias de bioconservación como las bacteriocinas, el sistema lactoperoxidasa (SLP) y la lactoferrina (LF), en un intento de potenciar posibles sinergias y minimizar la intensidad de los tratamientos aplicados sobre dos productos cárnicos RTE, jamón curado loncheado y carpaccio de ternera. Se han evaluado además, los cambios producidos como consecuencia de los tratamientos en las características físicoquímicas, reológicas y el color de estos productos.

Se aplicaron tratamientos individuales de alta presión a 400, 500 y 600 MPa durante 5 min sobre jamón curado loncheado inoculado con *S. enterica* serovar Enteritidis y mantenido en refrigeración durante 60 d a 8 ºC. Los tratamientos resultaron efectivos en la inactivación del patógeno. Las células dañadas no fueron capaces de recuperarse durante el almacenamiento, incluso en las condiciones de abuso de temperatura ensayadas. La oxidación lipídica aumentó con la presurización y el almacenamiento, mientras que el contenido en aminoácidos libres fue similar en las muestras control y en las tratadas por APH después de 60 d a 8 ºC. La resistencia al corte disminuyó con los tratamientos más intensos y la dureza cambió ligeramente. El color resultó afectado por la presurización, pero en general, los cambios inducidos por las APH se atenuaron durante el almacenamiento.

Los tratamientos de presurización a 400 y 500 MPa durante 10 min resultaron efectivos en la inactivación de *E. coli* O157:H7 inoculado en jamón curado loncheado, aunque las reducciones alcanzadas fueron pequeñas. Los tratamientos con nisina o pediocina no afectaron a la supervivencia del patógeno. Sin embargo, con tratamientos combinados de APH y nisina se observó un ligero efecto antimicrobiano sinérgico. La sinergia entre 500 MPa y nisina se mantuvo durante 60 d a 8 ºC,

proporcionando un mayor margen de seguridad en el control de este patógeno durante la refrigeración. La presurización combinada con los bioconservantes modificó algunos aspectos de la calidad del jamón, como la textura y el color.

Se aplicaron tratamientos de APH a 450 MPa durante 10 min en combinación con el SLP o LF sobre *L. monocytogenes* y *S*. Enteritidis inoculadas en jamón curado loncheado y almacenado durante 60 d a 8 ºC. *L. monocytogenes* mostró mayor resistencia al tratamiento de APH y la aplicación individual de los bioconservantes no afectó a los recuentos de ambos patógenos. Sin embargo, la combinación de APH y el SLP o LF produjo un efecto antimicrobiano sinérgico sobre *S*. Enteritidis con reducciones aproximadamente 2 unidades logarítmicas superiores a las alcanzadas con la suma de los efectos individuales. En el caso de *L. monocytogenes*, se detectó un ligero efecto antimicrobiano sinérgico con las APH en combinación con el SLP al final del período investigado. Se registraron niveles menores de viables totales en todas las muestras tratadas. La presurización modificó ligeramente la textura y el color del jamón curado.

En el carpaccio de ternera inoculado con *S*. Enteritidis se ensayaron tratamientos de 450 MPa durante 5, 10 y 15 min y el producto se mantuvo durante 30 d a 8 ºC. Los tratamientos resultaron eficaces en la inactivación del patógeno y el efecto antimicrobiano se mantuvo durante 30 d en condiciones de abuso de temperatura. Las APH afectaron ligeramente al color, aunque este efecto fue mayor con los tiempos de presurización más altos y la dureza aumentó al final del período investigado.

Cuando se investigó la supervivencia de los tres patógenos en carpaccio de ternera con tratamientos de 450 MPa durante 5 min se consiguieron reducciones de entre 1 y 3 unidades logarítmicas en los niveles de *L. monocytogenes*, *S*. Enteritidis y *E. coli* O157:H7. No se detectó actividad antimicrobiana del SLP ni de la LF activada (ALF). Se registró un efecto antimicrobiano sinérgico de las APH en combinación con el SLP frente a *S*. Enteritidis y *E. coli* O157:H7. Los recuentos de viables totales fueron menores en las muestras tratadas que en las control. El color y la textura resultaron ligeramente afectados por los tratamientos.

Con la combinación de presurización a 450 MPa durante 5 min y bacteriocinas de bacterias lácticas frente a *L. monocytogenes*, *S*. Enteritidis y *E. coli* O157:H7 en carpaccio de ternera, se comprobó que las bacteriocinas individualmente solo afectaron a *L. monocytogenes*. El efecto de la presurización combinado con nisina Z o pediocina comercial fue ligeramente sinérgico sobre *L. monocytogenes*. Con la

combinación de las APH y enterocinas A y B, pediocina PA-1 o nisina comercial, se detectó un efecto antimicrobiano sinérgico sobre *E. coli* O157:H7 con tasas de inactivación 2 unidades logarítmicas superiores a las alcanzadas con la suma de los efectos individuales. La nisina comercial redujo los viables totales y la combinación de APH con la pediocina o la nisina comercial produjo un efecto antimicrobiano sinérgico. El color y la textura se vieron ligeramente afectados.

Por último, con objeto de investigar nuevas estrategias, se ensayaron tratamientos combinados de APH y nitrito sódico para la inactivación de *L. monocytogenes* y *E. coli*. La combinación de 225 MPa y 300 MPa durante 8 min con nitrito acidificado fue sinérgica sobre la inactivación de *L. monocytogenes* y *E. coli*, respectivamente. La actividad bactericida del nitrito sódico fue dependiente del pH. Los mutantes *hmp*A y *nor*V de *E. coli* BW25113, carentes de enzimas protectoras frente al óxido nítrico, resultaron más sensibles al nitrito sódico acidificado y al tratamiento de APH que la cepa parental. Estas combinaciones servirán de base para futuros estudios en productos cárnicos, en un intento de disminuir la cantidad de nitritos añadidos en alimentos.

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- Uso de altas presiones y bioconservación para garantizar la seguridad microbiológica de alimentos listos para el consumo (RTE) (AGL2007-65235-C02-01/ALI)

- Productos cárnicos para el siglo XXI: seguros, nutritivos y saludables (CSD2007-00016)

- Antimicrobianos biológicos en seguridad alimentaria: reuterina, sistema lactoperoxidasa y lactoferrina (AGL2010-16600)

Consumers´current trends preferring less treated and safer food, with a longer shelf-life and easier to prepare, have increased the demand for ready-to-eat (RTE) products. These products which are directly consumed without higienization treatment or minimum process, can be contaminated during their manufacture/processing with pathogen microorganisms. *Listeria monocytogenes*, several *Salmonella* spp. serovars and *Escherichia coli* (including O157:H7) might contaminate these products during their manufacture and could become a risk for consumers, especially in temperature abuse conditions.

In the present study HHP has been chosen as a non-thermal preservation technology aimed to enhance microbiological safety and food shelf-life. HHP has been combined with several biopreservation strategies, namely bacteriocins, the lactoperoxidase system (LPS) and lactoferrin (LF), in an attempt to not only potentiate possible synergistic effects but also to minimize the severity of treatments conditions on two RTE meat products: sliced dry-cured ham and beef carpaccio. Moreover, the effect of the above mentioned technologies on the physicochemical and rheological characteristics as well as on the color of both meat products has been assessed.

High pressure treatments at 400, 500 and 600 MPa for 5 min were effective to inactivate *S. enterica* ser. Enteritidis inoculated on sliced dry-cured ham stored for 60 d at 8 ºC. Damaged cells were not able to recover during storage, even under temperature abuse conditions. Lipid oxidation increased with pressurization intensity and time of storage, whereas free amino acids content was similar in control and pressurized samples after 60 d at 8 ºC. Shear force decreased as pressure increased and hardness slightly changed. Color was affected by pressurization, but in general, those changes were attenuated during storage.

High pressure treatments at 400 or 500 MPa for 10 min were effective to inactivate *E. coli* O157:H7 inoculated on dry-cured ham, although the reductions achieved were low. The application of nisin or pediocin alone did not affect the pathogen survival. However, a slightly synergistic antimicrobial effect was detected with HHP and nisin combined treatments. The synergy achieved with 500 MPa and nisin persisted during the 60 d period at 8 ºC, providing a wider margin of safety in the control of this pathogen during refrigeration. Nevertheless, the combination of pressurization and biopreservatives modified some aspects of dry-cured ham quality, such as texture and color.

HHP at 450 MPa for 10 min in combination with the LPS or LF were applied against *L. monocytogenes* and *S*. Enteritidis inoculated on sliced dry-cured ham stored for 60 d at 8 ºC. *L. monocytogenes* showed higher resistance to HHP and the application of

biopreservatives alone did not affect both pathogen counts. In case of *L. monocytogenes*, a slightly synergistic antimicrobial effect was detected when HHP and the LPS were combined at the end of refrigerated period. However, the combination of HHP and the LPS or LF produced a synergistic antimicrobial effect against *S*. Enteritidis with inactivation rates of approximately 2 log units higher than those achieved by adding both individual effects. Lower levels of total viable counts (TVC) were detected in all treated samples. Pressurization slightly modified the texture and color of dry-cured ham.

Treatments at 450 MPa for 5, 10 or 15 min were assessed in beef carpaccio inoculated with *S*. Enteritidis and stored for 30 d at 8 ºC. Treatments were effective on the inactivation of the pathogen and the antimicrobial effect persisted during the 30 d period under temperature abuse conditions. HHP slightly affected color, being this effect more marked for longer holding times. Hardness was also affected, increasing at the end of the refrigerated period.

Treatments at 450 MPa for 5 min reduced *L. monocytogenes*, *S*. Enteritidis and also *E. coli* O157:H7 between 1 and 3 log units in beef carpaccio. No antimicrobial activity was detected when the LPS or activated LF (ALF) were applied alone. Synergistic antimicrobial effects were detected when HHP and the LPS were combined against *S*. Enteritidis and *E. coli* O157:H7. TVC levels were lower in treated than in non-treated samples. Color and texture were slightly affected by treatments.

When HHP at 450 MPa for 5 min and bacteriocins were combined against *L. monocytogenes*, *S*. Enteritidis and *E. coli* O157:H7 inoculated on beef carpaccio, it was observed that bacteriocins were only effective against *L. monocytogenes*. The antimicrobial effect of pressurization combined with nisin Z or a commercial pediocin preparation was slightly synergistic against *L. monocytogenes*. A synergistic antimicrobial effect was detected against *E. coli* O157:H7 when HHP and enterocins A and B, pediocin PA-1 or a commercial nisin preparation were combined. Inactivation rates were approximately 2 log units higher than those obtained by adding each single treatment. Commercial nisin reduced TVC and the combination of HHP with commercial nisin or pediocin preparations produced a synergistic antimicrobial effect against this microbial group. Color and texture were slightly modified.

The effect of combining sodium nitrite and high pressure on the inactivation of *L. monocytogenes* and *E. coli* was also investigated in a buffer system. The combination of acidified nitrite (pH 4.0) and 225 MPa or 300 MPa for 8 min was synergistic on *L. monocytogenes* and *E. coli*, respectively. The bactericidal activity of sodium nitrite was pH dependent. The *E. coli* BW25113 *hmp*A and *nor*V mutants, lacking the

protective enzymes against nitric oxide, resulted to be more sensitive to acidified sodium nitrite and HHP than wild-type *E. coli*. These combinations will be used in future studies on meat products, in an attempt to decrease the amount of nitrites added to foods.

1. Introducción

1. INTRODUCCIÓN

El estilo de vida de la sociedad actual en los países desarrollados ha originado una serie de cambios en los hábitos alimentarios de los consumidores. La tendencia hacia alimentos menos tratados pero más estables y seguros, con una vida útil más larga, más fáciles de preparar, nutritivos y elaborados sin la adición de conservantes químicos, puede conducir a una pérdida de la conservación intrínseca de los alimentos y de la protección a través del procesado. Las novedades introducidas en el procesado de los alimentos para dar respuesta a estas demandas pueden originar cambios en la microbiología de los productos y en su contaminación con microorganismos patógenos. Esto obliga a la industria alimentaria a ampliar los conocimientos sobre los patógenos que pueden contaminar sus productos, su origen y cómo se ven afectados por los nuevos procesos industriales.

Atendiendo a las demandas de los consumidores, la industria alimentaria ha transformado alimentos ya procesados (jamón serrano, jamón cocido, embutidos, fiambre de ave, pescado ahumado, quesos frescos y curados, etc.) o frescos (carne o pescado, etc.) en productos listos para el consumo (o RTE, del inglés Ready-To-Eat). Según el Reglamento 2073/2005, los alimentos listos para el consumo se definen como aquellos alimentos destinados al consumo humano directo sin necesidad de cocinado u otro tipo de transformación eficaz para eliminar o reducir a un nivel aceptable los microorganismos peligrosos (CE, 2005). Estos alimentos se presentan al consumidor en forma de lonchas, filetes, rodajas, piezas pequeñas, etc., envasados en porciones individuales o familiares. En la mayoría de los casos, los productos RTE son envasados a vacío o en atmósferas modificadas/protectoras y han de mantenerse en refrigeración hasta su consumo con el fin de alargar su vida útil. El objetivo del envasado a vacío es reducir el volumen de aire residual en contacto con el alimento. En estas condiciones, la pequeña cantidad de oxígeno que permanecería en el interior del envase desaparecería al ser consumido rápidamente por el propio producto o por los microorganismos capaces de desarrollarse en condiciones de microaerofilia. Así, mediante el envasado a vacío, se inhibe la microbiota aeróbica Gram-negativa (*Pseudomonas*, *Acinetobacter* y *Psychrobacte*r) y se seleccionan microorganismos anaerobios facultativos, como las bacterias ácido lácticas (BAL), pertenecientes fundamentalmente a los géneros *Lactobacillus*, *Carnobacterium* y *Leuconostoc*. Sin embargo, este envasado no proporciona una absoluta seguridad frente a los microorganismos patógenos cuando la temperatura es superior a 6º C, ya que, aunque las condiciones de vacío y presencia de CO_2 detienen y ralentizan la multiplicación de estafilococos y salmonellas, así como la producción de enterotoxinas estafilocócicas, en el caso de *Clostridium botulinum* y *C. perfringens*,

solamente un riguroso control de temperatura puede ser eficaz. Por otro lado, las atmósferas modificadas en los envases contienen una mezcla gaseosa con pequeñas concentraciones de oxígeno y concentraciones más elevadas de CO_2, aunque también pueden contener una determinada cantidad de gas(es) inerte(s) como el nitrógeno, a fin de mantener las concentraciones requeridas de los dos gases anteriores. El tipo de microbiota dominante dependerá de la composición gaseosa. Si se emplean niveles altos de oxígeno, se favorecerá el desarrollo de microorganismos aerobios. Si se usa una cantidad más baja de oxígeno y más alta de CO_2, se favorecerá el desarrollo de microorganismos Gram-positivos como las BAL y *Brochothrix thermosphacta*, en detrimento de bacterias Gram-negativas, más sensibles a la acción del CO_2.

Las formas de procesado de los alimentos RTE que implican una reducción de tamaño y conllevan la manipulación del alimento en operaciones como el loncheado, troceado, dosificación y envasado, incrementan los riesgos de una contaminación accidental, que puede verse acentuada si el almacenamiento de estos productos se realiza en condiciones de abuso, o aumento incontrolado de temperatura (Cava *et al.*, 2009; Garrido *et al.*, 2010).

Los alimentos RTE pueden contaminarse durante su preparación con microorganismos patógenos, procedentes del ambiente, utillaje empleado en las operaciones, manipuladores, etc. Por este motivo, debe mantenerse en todo momento la cadena de frío y deben manipularse estos alimentos siguiendo las normas higiénicas correspondientes. Los microorganismos patógenos más frecuentes son diversos serovares de *Escherichia coli* (incluido el O157:H7) y de *Salmonella* spp., *Campylobacter jejuni*, *Listeria monocytogenes*, *Yersinia enterocolitica* y *Staphylococcus aureus*. Entre estos patógenos, *L. monocytogenes* y *Salmonella* spp. pueden considerarse ubicuos por lo que se detectan con cierta frecuencia en una gran variedad de alimentos. *L. monocytogenes* es el patógeno que más preocupa entre los que se multiplican incluso en condiciones de refrigeración estricta, sin sobrepasar 4 ºC. *E. coli* O157:H7 posee una dosis infectiva muy baja y es responsable de un proceso patológico muy severo. *S. aureus* es un microorganismo oportunista, cuyo crecimiento en un alimento mantenido en refrigeración suele quedar condicionado a un abuso de la temperatura de almacenamiento.

Hay que tener en cuenta que el nivel de seguridad microbiológica de una porción de producto alimenticio en el momento del consumo es consecuencia de diversos factores, entre los que se encuentran la presencia de microorganismos en las diferentes etapas de producción, mezclado y división de los lotes, el crecimiento e

inactivación de los microorganismos, así como las combinaciones de temperatura y tiempo durante el procesado y el almacenamiento. El tamaño de la unidad de consumo, el método empleado para servir el alimento, la frecuencia de consumo, la relación dosis-respuesta y la susceptibilidad del consumidor son factores que afectan al riesgo microbiológico asociado al alimento. Por esta complejidad de factores, la seguridad de un alimento en la etapa de consumo depende de múltiples eventos y decisiones que se han producido o tomado a lo largo de toda la cadena alimentaria.

La implantación de buenas prácticas de fabricación y la aplicación de los principios del sistema de análisis de peligros y puntos de control críticos (APPCC) deben garantizar la seguridad de los alimentos. Sin embargo, pueden no resultar suficientes. La aplicación de tecnologías no térmicas como las altas presiones hidrostáticas (APH), los pulsos de luz, las radiaciones, etc., y determinados agentes biológicos (solos o en combinación con alguna de las tecnologías anteriormente mencionadas) como los ácidos orgánicos, las bacteriocinas y otros agentes antimicrobianos, se presentan como una herramienta eficaz para conseguir la higienización de los alimentos RTE.

1.1. MICROORGANISMOS PATÓGENOS TRANSMITIDOS POR LOS ALIMENTOS

Las bacterias patógenas que pueden contaminar alimentos proceden de diversos orígenes, no limitados exclusivamente a las materias primas alimentarias. Aunque algunas de estas bacterias patógenas son agentes de zoonosis, como *Salmonella* o *Listeria*, su presencia en los alimentos no se debe exclusivamente a la transmisión desde los animales portadores. Los manipuladores de alimentos también son una fuente de microorganismos patógenos, que pueden portar de forma asintomática, como consecuencia de una infección o por contaminación a partir de otros focos.

Las enfermedades microbianas de transmisión alimentaria pueden clasificarse en tres categorías (Sheryl, 2006):

- Infección invasiva, en la que el microorganismo invade y penetra la mucosa intestinal.
- Toxiinfección, en la que el microorganismo produce toxina durante su paso por el tracto intestinal.
- Intoxicación, en la que el microorganismo produce toxinas específicas o compuestos tóxicos en el alimento.

Se indican a continuación las características de los patógenos objeto de estudio en la presente tesis.

1.1.1. Bacterias patógenas Gram-negativas

Una parte importante de las bacterias Gram-negativas presentes en el intestino humano y de los animales se incluyen dentro de la familia *Enterobacteriaceae. Salmonella* y *Escherichia* son géneros de bacterias pertenecientes a esta familia que pueden causar enfermedades de transmisión alimentaria.

1.1.1.1. *Salmonella*

Las bacterias del género *Salmonella* son bacilos móviles, no esporulados y anaerobios facultativos (Le Minor, 1984). Su temperatura óptima de crecimiento es de 37 ºC, aunque depende de las cepas, y es capaz de crecer entre los 5 y los 47 ºC. El pH óptimo es de 7.0, pero tolera condiciones moderadamente ácidas, de pH 4.0 a 5.4. A pH bajo, el nitrito y el cloruro sódico son inhibidores de crecimiento. Valores de actividad de agua (a_w) superiores a 0.95 favorecen su desarrollo. *Salmonella* se mantiene sin dificultad en los alimentos desecados, sobre todo si son ricos en grasas y proteínas, con efecto protector. No es un microorganismo termotolerante y se destruye con un cocinado completo. *Salmonella* se distingue del resto de microorganismos causantes de toxiinfecciones alimentarias por su frecuencia en ciertos alimentos de consumo habitual, como huevos y productos derivados, productos de panadería, carnes de pollo, ternera, pavo y cerdo, etc. (EFSA, 2013). Además, cualquier alimento que presente contaminación fecal puede verse implicado, sobre todo los productos crudos o poco cocinados. Posee capacidad para multiplicarse en una amplia variedad de alimentos en un amplio intervalo de temperaturas, facilidad de diseminación de persona a persona y resistencia a las condiciones ambientales teóricamente adversas. El reservorio principal de *Salmonella* es el tracto intestinal de una amplia variedad de animales salvajes y domésticos. La transmisión tiene lugar cuando los microorganismos son introducidos en áreas de preparación de alimentos, multiplicándose en los mismos debido a temperaturas de almacenamiento inadecuadas, cocinado insuficiente o contaminación cruzada de alimentos RTE.

Salmonella produce en los humanos dos tipos de enfermedades de transmisión alimentaria: la salmonelosis y las fiebres tifoideas y paratifoideas. La salmonelosis es una enfermedad provocada principalmente por *S. enterica* serovar Typhimurium o *S. enterica* serovar Enteritidis (WHO, 1995; D'Aoust, 2000). Estas cepas se encuentran en el intestino de los animales, incluidos los de abasto. El patógeno se transmite al medio ambiente y a los alimentos a través de las heces. La transmisión fecal-oral es la más común, pero también puede ser persona-persona, sobre todo en colectividades. En cuanto al segundo tipo, las fiebres tifoideas y paratifoideas son causadas por *S. enterica* serovar Typhi y *S. enterica* serovar Paratyphi,

respectivamente (FDA/CFSAN, 1992a). Estos microorganismos aparecen en las heces humanas y se extienden al medio ambiente y a los alimentos, siendo también común la transmisión persona-persona.

La salmonelosis de origen alimentario se caracteriza por trastornos gastrointestinales, que se manifiestan sobre todo con diarreas y calambres intestinales. El período de incubación es de 6 a 48 h (Yoshikawa, 1980) y la enfermedad cursa con síntomas de fiebre moderada, náuseas, vómitos, dolor de cabeza, dolor en las extremidades, dolor abdominal y diarrea, que dura desde unos pocos días hasta una semana. La enfermedad puede ser grave en niños, ancianos y en personas inmunodeprimidas (FDA/CFSAN, 1992a; WHO, 1995). Los pacientes pueden convertirse en portadores asintomáticos por períodos de hasta 6 meses.

Para producir la enfermedad se requiere ingerir una cantidad mayor de 10^5 ufc. Sin embargo, hay algunas cepas virulentas que con pocas células pueden originar la enfermedad. Las cepas que son sensibles a la acidez gástrica, por lo general necesitan más células para poder establecerse en el intestino y ocasionar el trastorno. La cantidad puede ser menor si el patógeno se consume con alimentos que neutralizan la acidez estomacal, como leche, quesos, etc. La progresión de la enfermedad también depende del estado fisiológico del huésped; las personas ancianas con alto pH gástrico son más vulnerables a la salmonelosis. Después de la ingestión, el patógeno coloniza el intestino, adheriéndose a las células de la mucosa por medio de fimbrias y de otros factores de adhesión e invade de forma activa las células de la mucosa. Asimismo, la bacteria puede entrar por las células M de las placas de Peyer, que se localizan en el tejido linfoide del intestino delgado. *Salmonella* se multiplica dentro de las células epiteliales y los macrófagos, hasta provocar la lisis celular. Como resultado, se produce inflamación y edema intenso en el sitio de infección, lo que ocasiona daño a la mucosa. Las células inflamatorias activan a los macrófagos, liberando de manera progresiva prostaglandinas que incrementan los niveles de AMPc (adenosín-monofostato cíclico) en las células de la mucosa, lo que inhibe la captación de Na^+ y libera iones de Cl^-. El desequilibrio electrolítico ocasionado facilita la pérdida de líquido, lo que propicia la diarrea.

1.1.1.2. *Escherichia coli O157*

Escherichia coli O157 es una bacteria productora de toxiinfecciones que da lugar a enfermedades entéricas y/o sistémicas. Aunque no es muy frecuente, la infección puede ser muy grave y derivar en colitis hemorrágica (CH), síndrome urémico hemolítico (SUH) o púrpura trombótica trombocitopénica (PTT).

El reservorio principal de las cepas de *E. coli* O157 productoras de toxina shiga (stx) es el intestino del ganado de abasto, principalmente ganado vacuno (Chapman *et al.*, 1992), pero también ovejas, ciervos, cabras, aves de abasto, perros, ratas, moscas, pájaros y humanos pueden actuar como reservorio, siendo portadores asintomáticos. El ganado vacuno también puede ser portador de otras cepas de *E. coli* productoras de stx que no tienen tanta incidencia como patógenos humanos de transmisión alimentaria. La vía más común de extensión de la infección por *E. coli* O157 es el contacto persona-persona, pero también se transmite de los animales a las personas o por el consumo de alimentos contaminados.

E. coli O157 es un bacilo no esporulado, anaerobio facultativo, casi siempre móvil (Orskov, 1984). Pertenece a la familia *Enterobacteriaceae*, creciendo de forma óptima a 37 ºC y pH 7.0 (FDA/CFSAN, 1992b). No es capaz de multiplicarse a valores de a_w inferiores a 0.98. Crece a temperaturas de 7-8 ºC, en un intervalo de pH de 4.4 a 9.0 y en concentraciones de 6.5% de NaCl. Tiene una resistencia a las condiciones ácidas similar a otros serovares de *E. coli*, que en general son bastante resistentes. *E. coli* O157 no es termorresistente, se destruye durante el cocinado de los alimentos. En cuanto a su persistencia en el medio ambiente, se mantiene viable en los efluentes ganaderos y en los suelos durante meses.

El período de incubación de la enfermedad suele ser de 3 a 8 días, oscilando entre 1 y 11 días (FDA/CFSAN, 1992b). La dosis infectiva es muy baja, entre 10 y 100 ufc. Los síntomas de la infección son diversos: diarrea acuosa y colitis hemorrágica, acompañada de dolor abdominal agudo y a veces de vómitos. Puede progresar a SUH, con fallo renal, leucopenia y anemia (Nataro y Kaper, 1998). La PTT tiene unos síntomas similares al SUH, pero afectando también al sistema nervioso central (SNC), pudiendo producirse hemorragias internas y coágulos en el cerebro. Los niños pequeños tienen mayor probabilidad de desarrollar el SUH, que normalmente dura varios días o semanas y precisa hospitalización, incluyendo transfusiones de sangre y diálisis. Otras consecuencias más graves (coma) o fatales pueden afectar a grupos de población más susceptibles de niños (SUH) o ancianos (PTT) (Nataro y Kaper, 1998).

Las bacterias, una vez ingeridas, se adhieren al intestino grueso donde se multiplican y producen stx 1 y/o stx 2. Las stx pueden alcanzar los órganos que se van a ver afectados (riñón, SNC) y otras células que tengan receptores para estas toxinas. En las células, las stx se unen a los receptores específicos de la superficie celular, traspasan la membrana hasta el retículo endoplásmico e interfieren con la síntesis proteica, conduciendo a la muerte celular.

Debido a su baja dosis infectiva, no se requiere la multiplicación de la bacteria en los alimentos. Se ha demostrado la transmisión a través de alimentos como las carnes, sobre todo carnes picadas cocinadas insuficientemente (FDA/CFSAN, 1992b). También algunos alimentos listos para el consumo se han visto implicados en brotes, entre ellos salami y carnes cocidas, así como zumo de manzana no pasteurizado, leche cruda, queso elaborado con leche cruda, yogur y brotes de vegetales para ensalada (EFSA, 2013).

1.1.2. Bacterias patógenas Gram-positivas

1.1.2.1. *Listeria monocytogenes*

Listeria monocytogenes es una bacteria ubicua en el ambiente, encontrándose en las industrias agroalimentarias, cámaras frías y desagües, suelos, agua, efluentes, polvo y tejidos vegetales (Seeliger y Jones, 1986). Produce una infección invasiva denominada listeriosis, enfermedad que afecta a los humanos y a los animales de abasto (se cree que por la presencia de este patógeno en ensilados mal fermentados), aunque en el hombre también puede cursar como infección no invasiva.

Las bacterias de esta especie son bacilos, móviles a 25 ºC, pero que no suelen mostrar movilidad a 37 ºC (Seeliger y Jones, 1986). Son microorganismos anaerobios facultativos, con preferencia por ambientes microaerófilos cuando hay oxígeno. Pueden multiplicarse en alimentos a temperaturas de -1.5 ºC y se ha comprobado *in vitro* su capacidad de crecer a 45 ºC. Se multiplica en un intervalo de pH de 4.1 a 9.6 y en concentraciones de 10% de NaCl. *L. monocytogenes* es un microorganismo muy resistente a la desecación, produce biofilms muy persistentes en las industrias agroalimentarias y puede sobrevivir hasta 1 año en concentraciones del 16 % de NaCl.

Las dos formas de listeriosis humana pueden ser transmitidas por alimentos. Entre los síntomas de la listeriosis invasiva, se encuentran septicemia, meningitis, encefalitis y abortos espontáneos (FDA/CFSAN, 1992c). Las poblaciones de mayor riesgo son mujeres embarazadas, pacientes inmunocomprometidos, con SIDA, cáncer, niños, ancianos, pacientes con enfermedades cardíacas y hepáticas (Farber y Peterkin, 2000). En las mujeres embarazadas, los abortos suelen ocurrir en el tercer trimestre de gestación, provocando la muerte del feto. La listeriosis no invasiva tiene un período de incubación corto (1 a 3 días) y cursa con síntomas entéricos, como diarrea, fiebre moderada, dolor de cabeza y mialgia. Puede afectar a individuos sanos, pero no es muy probable, ya que dada su ubicuidad, *L. monocytogenes* es

ingerida regularmente por estos individuos con los alimentos sin producir síntomas de enfermedad.

En la listeriosis invasiva, *L. monocytogenes* atraviesa el epitelio intestinal, posiblemente por las células del epitelio o de las placas de Peyer (Farber y Peterkin, 2000). La bacteria se transporta a través de la sangre y del sistema linfático hasta el hígado y el bazo, siendo eliminada casi en su totalidad por los macrófagos. Sin embargo, cuando la respuesta inmunitaria en el hígado no es la adecuada, las células bacterianas supervivientes se multiplican intracelularmente en los macrófagos y comienza su diseminación de unas células a otras, provocando la muerte celular y extendiéndose al SNC, el corazón, los ojos o el feto. Posiblemente, en la listeriosis no invasiva, la acción de los macrófagos elimina totalmente al microorganismo.

La dosis infectiva no se conoce y se estima que está muy relacionada con la virulencia de la cepa, aunque en los individuos inmunocomprometidos se sitúa entre 100 a 1000 ufc, por lo que no sería necesaria la multiplicación del microorganismo en los alimentos.

En la listeriosis no invasiva, la dosis infectiva es más elevada ($>10^5$ ufc) y es importante controlar la multiplicación del microorganismo en los alimentos. Algunos alimentos implicados en brotes de esta enfermedad (EFSA, 2013) son el queso fresco y el queso blando, la leche cruda y pasteurizada, alimentos RTE, productos elaborados con carne de ave (salchichas de pavo), productos de la pesca, y algunos vegetales y ensaladas.

Según los criterios de seguridad alimentaria para los distintos patógenos, el patrón universal para *Salmonella* spp. y *E. coli* O157:H7 es de "tolerancia cero" (ausencia en 25 g). Sin embargo, el criterio para *L. monocytogenes* en alimentos RTE difiere entre países. Para el Departamento de Agricultura de EE.UU. (USDA) es de la categoría "tolerancia cero" pero para la Unión Europea (CE, 2007) varía dependiendo de la población receptora y del tipo de alimento. Para la población normal, el criterio es de 100 ufc/g siempre que las listerias no se multipliquen durante el almacenamiento. El reglamento de la UE considera que pertenecen a esta categoría, los productos RTE de pH≤4.4, los de a_w≤0.92, los de pH≤5.0 y a_w≤0.94 y los de vida útil inferior a 5 días.

En la UE y por lo tanto en España, los criterios microbiológicos vigentes se rigen por el Reglamento (CE) 2073/2005 (CE, 2005) así como por sus sucesivas modificaciones: el Reglamento (CE) 1441/2007 (CE, 2007), el Reglamento (UE) 365/2010 (UE, 2010) y el Reglamento (UE) 1086/2011 (UE, 2011).

1.1.3. Incidencia de *Salmonella* spp., *E. coli* y *L. monocytogenes* en la Unión Europea

Según el último informe de la Autoridad Europea de Seguridad Alimentaria (EFSA) publicado en 2013 (EFSA, 2013), la incidencia de *Salmonella* y *L. monocytogenes* ha disminuido durante los últimos años, a diferencia de *E. coli* verocitotoxigénico (VTEC) que ha aumentado desde 2008. A este respecto, hay que destacar el elevado número de casos acontecidos en la UE, principalmente en Alemania, en el año 2011 por el consumo de una mezcla de brotes contaminados con *E. coli* O104:H4 productor de shigatoxina (Buchholz *et al.*, 2011).

Los tres patógenos han sido detectados en una amplia variedad de alimentos, entre los que destacan la carne de pollo, cerdo, ternera, pavo y productos derivados, leche y productos lácteos, frutas, vegetales, productos de la pesca y alimentos RTE a base de carne (de consumo crudo) en el caso de *Salmonella*. En el caso de *E. coli* son las carnes de ternera y pollo, leche cruda de vaca y productos lácteos, quesos, mantequilla, brotes de semilla y otros vegetales y por último, en el caso de *Listeria*, fundamentalmente productos RTE de origen cárnico, de la pesca, embutidos fermentados y quesos.

1.2. TECNOLOGÍAS DE CONSERVACIÓN

La demanda de los consumidores de alimentos con menos conservantes y mínimamente procesados hace necesario el desarrollo de nuevas barreras u obstáculos para evitar el crecimiento de microorganismos patógenos. La combinación de técnicas tradicionales (temperatura, a_w, potencial redox (Eh), etc.) y otras estrategias de conservación (envasado en atmósferas modificadas, bacteriocinas, ácidos orgánicos, cultivos bioprotectores, APH, etc.), podrían establecer una serie de barreras más efectivas contra las bacterias alterantes y patógenas, sin afectar a la microbiota no alterante. En un determinado alimento se pueden utilizar múltiples barreras u obstáculos (Leistner, 2000) para controlar el comportamiento microbiano, pudiendo actuar de forma aditiva o sinérgica. Por lo tanto, cada parámetro adicional proporciona un nivel de control superior sobre el comportamiento microbiano (crecimiento, producción de toxinas, muerte, etc.). Aún cuando cada alimento o proceso nuevo o modificado originará nuevas condiciones para los microorganismos, utilizando el concepto de barreras u obstáculos, se deberán obtener alimentos más seguros para los consumidores.

En el presente trabajo se ha investigado en la combinación de las APH con otras estrategias de conservación como la bioconservación, en un intento de minimizar la intensidad de los tratamientos y los cambios indeseables que se pueden producir en

la carne y los productos cárnicos a presiones superiores de 400 MPa, además de aumentar la inactivación microbiana y reducir la recuperación de las células dañadas por las altas presiones.

1.2.1. ALTAS PRESIONES HIDROSTÁTICAS (APH)

El tratamiento por APH es un proceso físico no térmico que consiste en someter al alimento a una elevada presión. El fluido transmisor de la presión suele ser el agua, de ahí el nombre de APH, pero existen otros fluidos, de los cuales los más frecuentemente utilizados son el aceite de silicona, soluciones de benzoato sódico, propilenglicol y etanol. En los últimos años se ha renovado el interés de las APH como tecnología alternativa a los tratamientos térmicos para eliminar microorganismos alterantes y patógenos de los alimentos, ampliar su vida útil y mejorar la seguridad microbiológica, afectando mínimamente a sus propiedades organolépticas.

Los efectos de las altas presiones se fundamentan en dos principios:
- Principio de Le Chatelier, que indica que cualquier fenómeno que va acompañado de una disminución de volumen se verá potenciado cuando se aplica presión.
- Principio de Pascal, que predice que la presión aplicada en un punto a un fluido incompresible, contenido en un recipiente indeformable, se transmite de forma instantánea y homogénea, independientemente del tamaño y la geometría del medio. Es lo que se conoce como presión isostática.

El tratamiento de altas presiones es especialmente útil para aquellos alimentos con características funcionales y sensoriales sensibles al calor. La presión solo modifica enlaces no covalentes, por lo que no deteriora nutrientes termolábiles como vitaminas, ni altera los compuestos de bajo peso molecular responsables del sabor y del aroma (Smelt, 1998). Sin embargo, puede producir desnaturalización de proteínas, inactivación de enzimas, cambios en las interacciones sustrato-enzima, así como en los hidratos de carbono y las grasas (Butz y Tauscher, 2002).

Las cámaras de presurización para uso comercial suelen tener capacidades de 35 a 350 litros. Las presiones utilizadas habitualmente en la industria alimentaria se sitúan en el intervalo de 300 a 700 MPa (Ordóñez *et al.*, 2004).

La industria cárnica emplea las APH como tecnología post-envasado para aumentar la vida útil y mejorar la seguridad alimentaria de los productos cárnicos RTE. Su aplicación puede originar, en mayor o menor medida, modificaciones de algunos parámetros de calidad como el color, la textura y la capacidad de retención de agua, sin afectar negativamente su valor nutricional. Esta tecnología cuenta con un buen

grado de aceptación por parte del consumidor, lo que ha llevado a una mayor implantación a nivel industrial con respecto a otras tecnologías emergentes. Los niveles de intensidad de aplicación del tratamiento por la industria cárnica oscilan entre 400-600 MPa con tiempos cortos de presurización de entre 3 y 7 minutos a temperatura ambiente. Estas condiciones de tratamiento conducen, en la mayoría de los casos, a un nivel de inactivación de aproximadamente 4 unidades logarítmicas para la mayoría de los microorganismos patógenos y alterantes (Bajovic *et al.*, 2012).

1.2.1.1. Efecto de las APH sobre los microorganismos

La respuesta de los microorganismos patógenos y alterantes frente a las altas presiones depende de factores externos como la presión, la temperatura, el tiempo de aplicación del tratamiento y de otros parámetros como el pH, la a_w, el contenido en sal y la presencia de otros antimicrobianos en el alimento (Rendueles *et al.*, 2011). La composición química del alimento determina la resistencia o sensibilidad de los microorganismos al tratamiento de presurización, y la presencia de grasas, proteínas, minerales y azúcares actúan como agentes protectores frente a las altas presiones. También son determinantes factores intrínsecos como el tipo de microorganismo, el estado fisiológico y otras particularidades de las cepas (Jofré *et al.*, 2010), observándose una elevada variabilidad en la resistencia a las altas presiones, incluso entre cepas de la misma especie (Liu *et al.*, 2012). Los microorganismos Gram-negativos son más sensibles a las altas presiones que los Gram-positivos. La morfología celular también influye, siendo los bacilos más sensibles a la presurización que los cocos. En cuanto al estado fisiológico, los microorganismos en fase exponencial son más sensibles a las altas presiones que los que se encuentran en fase estacionaria (Mañas *et al.*, 2004).

Los mecanismos de inactivación bacteriana por la alta presión no se conocen completamente, pero el daño de membranas, la desnaturalización proteica y el estrés oxidativo parecen jugar un papel importante (Mackey *et al.*, 2008).

Se ha propuesto la pérdida de integridad de la membrana citoplasmática como uno de los eventos críticos que conducen a la muerte de los microorganismos tratados por presión, produciéndose un cambio de permeabilidad de la misma (Pagán y Mackey, 2000). Sin embargo, esta pérdida parcial de funcionalidad o el daño producido en la membrana, no necesariamente conducen a la muerte celular, ya que las células bacterianas que sobreviven pueden experimentar una mayor sensibilidad a sustancias inhibidoras, tales como cloruro sódico, sales biliares e ingredientes de medios selectivos (Mackey *et al.*, 2000). En algunos trabajos (Alpas *et al.*, 2000;

Jordan *et al.*, 2001; Chilton *et al.*, 2001) se ha demostrado la existencia de daño subletal después de la aplicación de tratamientos de alta presión usando técnicas de plaqueo diferencial. Los daños en la membrana causados por la presurización permiten la aplicación de tratamientos combinados para conseguir una mayor efectividad de las altas presiones (Alpas *et al.*, 2000; Masschalck *et al.*, 2003).

Respecto a la desnaturalización proteica, se ha observado que interacciones electrostáticas e hidrofóbicas en y entre proteínas son especialmente sensibles a la presión, haciendo que las estructuras terciaria y cuaternaria resulten afectadas por el tratamiento. Se ha demostrado que tanto la desnaturalización proteica como la disrupción de membrana en células bacterianas tratadas por presión, dan lugar a la inactivación de enzimas y procesos clave para el funcionamiento óptimo de las células (Ritz *et al.*, 2000).

Con respecto al estrés oxidativo, hay evidencia de que la presión puede conducir a la generación de especies reactivas del oxígeno (ROS) dentro de la célula. Los mecanismos no se conocen completamente pero la disrupción de los procesos respiratorios normales podría conducir a la formación de ROS (Aertsen *et al.*, 2005). La inactivación de superóxido dismutasa y/o catalasa desprotegen a las células de las especies ROS.

1.2.1.2. Efecto de las APH sobre las proteínas cárnicas

Las APH producen desnaturalización total o parcial de las proteínas, dependiendo de las condiciones aplicadas. Según el principio de Le Chatelier y Braun, las altas presiones favorecen las reacciones que conllevan una disminución de volumen. La formación de enlaces de hidrógeno implica una reducción de volumen y se ve por tanto favorecida por el aumento de la presión. A presiones bajas o moderadas (<100 MPa), la formación de enlaces de hidrógeno permite mantener la estructura helicoidal de las proteínas, minimizándose así los efectos de la presurización. Presiones del orden de 100-300 MPa producen una desnaturalización reversible de las proteínas y presiones superiores a 300 MPa provocan la desnaturalización irreversible de las mismas. La estructura cuaternaria de las proteínas, mantenida por interacciones hidrofóbicas, es muy sensible a la presión. En la estructura terciaria, los cambios importantes tienen lugar a presiones superiores a 200 MPa y en la estructura secundaria ocurren a presiones por encima de 700 MPa (Rastogi *et al.*, 2007).

Las proteínas musculares sufren desnaturalización, aglomeración y forman geles con tratamientos superiores a 300 MPa. La presurización modifica el complejo actina-miosina (Nishiwaki *et al.*, 1996).

1.2.1.3. Efecto de las APH sobre el color de la carne y de los productos cárnicos

El color de la carne depende fundamentalmente de la cantidad total de mioglobina que contenga y del equilibrio establecido en las capas superficiales entre las tres formas del pigmento base, oximioglobina, mioglobina y metamioglobina. Sin embargo, el color de los productos curados es atribuido a la presencia de nitrosilmioglobina, resultado de la reacción del óxido nítrico (formado a partir del nitrito/nitrato sódico) con la mioglobina. De diferentes investigaciones publicadas se desprende que las APH provocan cambios drásticos en el color de la carne fresca (Carlez *et al.*, 1995), mientras que en los productos crudos-curados estos cambios son aceptables y dependen del contenido de agua y del valor de a_w del producto (Ferrini *et al.*, 2012). Los tratamientos de 200-350 MPa aumentan los valores de luminosidad (L^*) en la carne fresca y dan lugar a un color rosáceo. La aplicación de tratamientos de 400-500 MPa (Carlez *et al.*, 1995) provoca una disminución de la tendencia al rojo (a^*). Diversos estudios llevados a cabo en productos cárnicos curados presurizados revelan un aumento en los valores de L^* y una disminución de los valores de a^*, acentuándose estas tendencias con un mayor contenido en agua de los productos cárnicos (Ferrini *et al.*, 2012; Bak *et al.*, 2012).

En general, los cambios inducidos en el color por las altas presiones varían en función de diferentes parámetros, entre ellos, el contenido de mioglobina, siendo estos cambios más intensos en carne roja fresca que en carne blanca y productos cárnicos curados. El efecto negativo sobre el color puede limitarse optimizando variables como la intensidad y el tiempo de presurización, la temperatura, el curado, la disponibilidad de oxígeno y el pH.

1.2.1.4. Efecto de las APH sobre la oxidación lipídica de la carne y de los productos cárnicos

Se ha comprobado que tratamientos de presurización de entre 300 y 600 MPa inducen oxidación lipídica en la carne de cerdo (Cheah y Ledward, 1996), carne de ternera (Ma *et al.*, 2007; McArdle *et al.*, 2010) y carne de pollo (Bragagnolo *et al.*, 2007) y este efecto es mayor que en los productos cárnicos curados presurizados (Clariana *et al.*, 2011). Parece que las altas presiones podrían por un lado, producir la ruptura de los grupos hemo de las hemoproteínas y liberar el hierro que promovería la oxidación lipídica, y por otro lado, al desintegrar las membranas, posibilitar que los ácidos grasos poliinsaturados sean más susceptibles a la oxidación.

1.2.1.5. Efecto de las APH sobre la textura de la carne y de los productos cárnicos

Las APH inducen modificaciones sobre la textura de la carne, al afectar a la estructura de las proteínas miofibrilares, provocando su desnaturalización y agregación y afectando a su propiedad de formar geles (Colmenero, 2002). En general, los tratamientos de presurización inferiores a 200 MPa pueden ablandar la carne antes de que tenga lugar el *rigor mortis* (pre-rigor), pero la tenderización o ablandamiento post-rigor mediante altas presiones solo puede alcanzarse mediante la combinación de la presurización con tratamientos térmicos, aplicando temperaturas de entre 40 y 80 ºC (Sun y Holley, 2010). La influencia de las altas presiones sobre la textura depende del estado rigor de la carne, de los niveles de presión y temperatura aplicados (Ma *et al.*, 2004) y de la duración del tratamiento (Sun y Holley, 2010). Se piensa que la tenderización por altas presiones puede ser causada por la rotura de los lisosomas y posterior actividad proteolítica de la catepsina D y de la fosfatasa ácida, que afectarían a la textura de la carne (Jung *et al.*, 2000).

1.2.2. BIOCONSERVACIÓN

El término de bioconservación (Stiles, 1996) hace referencia al incremento de la vida útil y de la seguridad de los alimentos utilizando la microbiota natural o controlada y/o sus productos antibacterianos.

1.2.2.1. Bacteriocinas

Las bacteriocinas son polipéptidos o proteínas cuyo tamaño oscila entre 900 y 5800 Daltons, sintetizadas en los ribosomas, que en su forma madura producen un efecto antibacteriano contra bacterias estrechamente relacionadas con las bacterias productoras. Las bacteriocinas son producidas por bacterias Gram-negativas y Gram-positivas. Pueden proporcionar a sus productores, inmunes a los productos antibacterianos que sintetizan, una ventaja adaptativa frente a otras bacterias. Generalmente, eliminan la fuerza protónica que atraviesa las membranas bacterianas, al formar pequeños poros en éstas, pero también pueden inhibir la síntesis de proteínas, la formación de ARN-m y la duplicación del ADN. Aunque las células de las bacterias Gram-negativas son resistentes a las bacteriocinas de las BAL, el estrés químico o físico puede deteriorar la estructura lipopolisacarídica de la membrana externa y hacerlas sensibles a la acción de las bacteriocinas.

En microbiología de alimentos, las bacteriocinas producidas por las cepas BAL son de especial interés porque normalmente dirigen su efecto bactericida a diferentes bacterias Gram-positivas alterantes y a bacterias patógenas, y bajo condiciones de

estrés químico (ácidos orgánicos, agentes quelantes, etc.) o físico (pH, congelación, APH, etc.) pueden dirigir su efecto bactericida contra diferentes Gram-negativas importantes para los alimentos (Fang y Tsai, 2003; Kalchayanand *et al.*, 1992, 1998; Stevens *et al.*, 1991). Sin embargo, hay que tener en cuenta que la producción de una bacteriocina en medios sintéticos de laboratorio no implica su efectividad en un sistema alimentario. La carne y los productos cárnicos son sistemas complejos en los que puede producirse una pérdida parcial o total de la actividad bacteriocina, pudiendo ésta verse afectada por el procesamiento o las condiciones de almacenamiento del alimento, por cambios en la solubilidad y en la carga, por unirse a componentes del alimento o por ser destruidas por proteasas (Aasen *et al.*, 2003).

Las bacteriocinas, debido a su naturaleza proteica, son probablemente inactivadas por proteasas en el tracto gastrointestinal, siendo en su mayoría, catiónicas y termoestables. Se han definido cinco clases de bacteriocinas (Klaenhammer, 1993; Cotter *et al.*, 2006; Maqueda *et al.*, 2004; Kawai *et al.*, 2004).

La bioconservación mediante bacteriocinas puede ser aplicada en alimentos y sistemas cárnicos por cuatro métodos básicos:

1.- Adición de cepas de BAL que crecen y producen sustancias antagonistas. Este método ofrece una manera indirecta de incorporar bacteriocinas en un producto alimentario. Su éxito depende de la capacidad del cultivo para crecer y producir bacteriocinas en el alimento bajo condiciones ambientales y tecnológicas (temperatura, pH, ingredientes, Eh, presencia de microorganismos competidores, etc.). Puesto que la carne no puede ser pasteurizada antes de añadirle un cultivo de BAL, éstas deben ser capaces de competir con la microbiota natural de la carne.

2.- Adición de sustancias antagonistas purificadas. Con este sistema, la dosificación de bacteriocinas es más precisa y por lo tanto, más predecible. No obstante, su aplicación queda limitada por las leyes nacionales de aditivos alimentarios.

3.- Adición del fermentado o un concentrado de un microorganismo productor. Este modo evita la utilización de un compuesto purificado y por lo tanto, la obligación de declarar su presencia en el etiquetado.

4.- Adición de BAL mesófilas como una protección fallo-seguro contra abusos de temperatura. En este caso, la cepa bioprotectora se mantendrá en las concentraciones iniciales en condiciones de refrigeración. Bajo condiciones de abuso de temperatura, la cepa crecerá de forma competitiva frente a las bacterias patógenas evitando riesgos de salud.

1.2.2.1.1. Nisina

La nisina es una bacteriocina producida por algunas cepas de *Lactococcus lactis* y *Streptococcus* sp. Es una proteína hidrofóbica de 34 aminoácidos y bajo peso molecular (5 kDa). Se han descrito diferentes tipos de nisina, entre ellas, la nisina A, Z, Q y F producidas por *L. lactis* y la nisina U y U2 producidas por *S. uberis* 42 y *S. agalactiae* D536, que difieren entre sí en algunos residuos de aminoácidos. La nisina no es tóxica para los seres humanos y es la única bacteriocina reconocida como GRAS (Generally Regarded As Safe) por la FDA (Food and Drug Administration, EE.UU.), permitiéndose su uso comercial (O´Keeffe y Farrell, 2000) en más de 50 países, incluyendo EE.UU. y algunos países de la Unión Europea. La nisina se incluye dentro de la lista de aditivos aprobados con el número E234 (95/2/EC), sin efectos adversos conocidos. Pertenece a la clase Ia dentro de la clasificación de Klaenhammer (1993) y evita la germinación de las esporas de Gram-positivos de los géneros *Clostridium* y *Bacillus* (Savadogo *et al.,* 2006), mostrando también eficacia frente a organismos Gram-positivos de los géneros *Staphylococcus*, *Listeria*, *Enterococcus*, etc. Se utiliza en quesos fundidos y carne enlatada, en queso cottage para inhibir el crecimiento de bacterias psicrotrofas, y en fermentaciones de cerveza y vino para prevenir el crecimiento de lactobacilos alterantes, entre otras aplicaciones.

La diana principal de la nisina es la membrana plasmática de las células bacterianas (Klaenhammer, 1993). Se inserta en ellas formando pequeños poros y la despolariza, altera su permeabilidad y promueve la liberación de moléculas de bajo peso molecular. Al mismo tiempo, afecta al transporte de nutrientes y la síntesis de ATP y finalmente, ocasiona la pérdida de viabilidad celular. El hecho de que la nisina sea activa a concentraciones muy bajas, del rango nanomolar, hizo plantearse la existencia de algún receptor específico para su unión a la membrana y el lípido II parece tener esa función (Wiedemann *et al.,* 2001). Al bloquearse la disponibilidad del lípido II queda inhibida la síntesis de la pared celular en la bacteria sensible, ya que es uno de los precursores esenciales.

En el mercado existen productos comercializados con actividad antimicrobiana constituidos por cultivos vivos de *L. lactis* productores de nisina.

1.2.2.1.2. Pediocina

La pediocina PA-1 es producida por *Pediococcus acidilactici*, aunque puede ser producida por otras especies del mismo género e incluso por diferentes géneros de BAL (Miller *et al.,* 2005). Pertenece a la clase IIa de las bacteriocinas (Klaenhammer, 1993) y es activa frente a algunas especies de los géneros *Lactococcus*,

Lactobacillus, *Carnobacterium*, *Enterococcus*, *Bacillus*, *Brochothrix*, *Clostridium*, *Listeria* y *Staphylococcus*, aunque la sensibilidad depende de la especie e incluso de la cepa. Presenta actividad inhibitoria frente a *L. monocytogenes* (Nielsen *et al.*, 1990).

La pediocina PA-1 es bactericida, como consecuencia de la formación de poros en la membrana citoplasmática, permeabilización de la misma y liberación de componentes intracelulares (iones, aminoácidos y otros compuestos de bajo peso molecular) (Deegan *et al.*, 2006). Se disipa la fuerza protón-motriz, reduciéndose o perdiéndose totalmente el potencial eléctrico transmembrana (Δψ) y el gradiente de protones (ΔpH) (Jack *et al.*, 1995). La síntesis de ATP y algunos sistemas de transporte resultan afectados. En el mercado existen productos comercializados a base de fermentados de un cultivo iniciador de *P. acidilactici* productor de pediocina, recomendados especialmente para productos cárnicos y lácteos. Estos preparados pueden emplearse como ingredientes porque alguno de los componentes del fermentado puede aportar alguna funcionalidad al alimento. Además, durante la fermentación se producen otros metabolitos como los ácidos orgánicos, con actividad antibacteriana (Gálvez *et al.*, 2007).

1.2.2.1.3. Enterocinas

Las enterocinas son bacteriocinas producidas por bacterias pertenecientes al género *Enterococcus*. Entre las enterocinas descritas, se encuentran las enterocinas A y B, AS-48, I, L50A y L50B, CCM 4231, 13, 416K1, etc. (Khan *et al.*, 2010).

Las enterocinas A y B son producidas por algunas cepas de *Enterococcus faecium* y pertenecen a la clase IIa de las bacteriocinas (Klaenhammer, 1993). La enterocina A fue aislada de la cepa *E. faecium* CTC 492 y la enterocina B de la cepa *E. faecium* T136. Ambas actúan sinérgicamente y son activas frente a un amplio espectro de bacterias Gram-positivas que incluyen especies de *Clostridium*, *Propionibacterium*, *Listeria*, *Staphylococcus* y la mayoría de BAL. Diferentes estudios han demostrado que las enterocinas A y B son efectivas frente a *L. monocytogenes*, *Salmonella* y *S. aureus* inoculados en productos cárnicos (Garriga *et al.*, 2002) y proporcionan protección extra cuando se rompe la cadena de frío (Marcos *et al.*, 2008).

La enterocina AS-48 es una bacteriocina circular producida por *E. faecalis* subsp. *liquefaciens* S-48, perteneciente a la clase V de las bacteriocinas y activa frente a especies de bacterias Gram-positivas pertenecientes a los géneros *Bacillus*, *Corynebacterium*, *Mycobacterium*, *Micrococcus*, *Staphylococcus* y algunas especies de Gram-negativas de los géneros *Salmonella*, *Shigella*, *Escherichia*, *Pseudomonas* y

Klebsiella (Gálvez *et al.*, 1989). Esta enterocina inactivó a *L. monocytogenes* y *S. aureus* en embutidos modelo (Ananou *et al.*, 2005a, 2005b).

La enterocina I es una bacteriocina producida por *E. faecium* 6T1a, cepa aislada originalmente de la fermentación de la aceituna verde estilo español. Es activa frente a bacterias alterantes y patógenas, entre las que se incluyen especies de los géneros *Clostridium*, *Propionibacterium* y *Listeria* (Floriano *et al.*, 1998).

1.2.2.2. Sistema lactoperoxidasa

El sistema lactoperoxidasa (SLP) forma parte de los mecanismos de defensa de la inmunidad innata en los mamíferos. Está presente en diferentes fluidos biológicos y está constituido por tres componentes: la enzima lactoperoxidasa (LPX), el ión tiocianato (SCN^-) y el peróxido de hidrógeno (H_2O_2), siendo estos dos últimos necesarios para su activación. La LPX es una glicoproteína de aproximadamente 78 kDa de peso molecular que contiene un grupo hemo (protoporfirina IX), pertenece al grupo de las peroxidasas, y protege a las células del metabolismo tóxico del oxígeno. Cataliza la oxidación del SCN^- por el H_2O_2, generando compuestos intermediarios activos con propiedades antimicrobianas, fundamentalmente, el ión hipotiocianito ($OSCN^-$), tiocianógeno ($(SCN)_2$), tiocianato cianógeno (NC-SCN), los aniones del ácido cianosulfuroso (HO_2SCN) y los del ácido cianosulfúrico (HO_3SCN). El metabolito principal es el $OSCN^-$, encontrándose en equilibrio con el ácido hipotiocianoso (HOSCN), con p*K*a = 5.3. Tanto el $OSCN^-$ como HOSCN son fuertes agentes oxidantes que ejercen su acción sobre grupos sulfhidrilos de enzimas y otras proteínas presentes en las membranas bacterianas.

Para activar el SLP es necesaria la presencia de H_2O_2 que puede ser generado enzimáticamente mediante la glucosa oxidasa (GOX). La GOX de *Aspergillus niger* es una glicoproteína bien caracterizada compuesta por un homodímero con dos subunidades idénticas de 80 kDa y dos coenzimas flavín-adenín-dinucleótidos, unidas no covalentemente, que actúan como transportadores de electrones durante la catálisis. Esta enzima cataliza la oxidación de β-D-glucosa ($C_6H_{12}O_6$) a D-gluconolactona ($C_6H_{10}O_6$) y H_2O_2 (Wilson y Turner, 1992), que se rompen espontánea y catalíticamente. La D-gluconolactona mediante la acción de una lactonasa, da lugar a ácido glucónico ($C_6H_{12}O_7$) que se acumula y reduce el pH de la solución. La presencia de GOX junto con su sustrato permite que el H_2O_2 requerido por el SLP sea generado de forma continua (Seifu *et al.*, 2005). Se ha demostrado que en presencia de catalasa, el H_2O_2 requerido para inhibir el crecimiento microbiano debe estar en niveles de mili-molar, mientras que en presencia de GOX, sería suficiente un nivel de micro-molar para ejercer su actividad inhibitoria (Leiter *et al.*, 2004). La GOX posee

el estatus GRAS según la FDA (FDA/CFSAN, 2002), se encuentra disponible en forma líquida o en polvo para su uso por la industria alimentaria y posee propiedades conservantes, estabilizantes y antioxidantes. El ácido glucónico (E574) está dentro de la lista de aditivos aprobados para uso alimentario en la Unión Europea (95/2/EC), utilizándose como regulador de la acidez, estabilizador del color, antioxidante y agente quelante (Codex Alimentarius Commission, 2007).

Por lo tanto, el sistema SLP-GOX estaría constituido por las enzimas LPX y GOX, glucosa y una fuente de iones isotiocianato. Su acción está influida por diversos factores, como la especie bacteriana, el pH del medio, el tiempo de incubación, la temperatura y la densidad celular (Björck *et al.*, 1975).

En el año 2002, la Autoridad Alimentaria de Australia y Nueva Zelanda elaboró un informe sobre el uso de la LPX y del NaSCNO/KSCNO (FSANZ, 2002) en la superficie de la carne, con el objetivo de reducir las poblaciones microbianas presentes. Para que el sistema SLP-GOX fuese efectivo, los niveles de los componentes individuales aplicados por Kg de carne, debían estar en los siguientes intervalos: LPX (800-2800 U), NaSCNO/KSCNO (30-40 mg), GOX (150-300 U) y glucosa (120-160 mg).

A los niveles propuestos, ni los componentes del SLP ni los productos intermediarios, poseen un riesgo significativo para la salud humana. Existen altos niveles de tiocianato de forma natural en la saliva humana y en el jugo gástrico y los compuestos intermediarios del tiocianato están presentes en la saliva, produciendo daño selectivo a la membrana bacteriana, pero no a las células eucariotas de los mamíferos. Solo los consumidores que presenten alergia a las proteínas lácteas deben ser informados mediante el etiquetado de la presencia de estas proteínas en la carne o en los productos cárnicos. El nivel de LPX que estaría presente sería bajo y según expertos, es poco probable que cause reacciones adversas, si tiene lugar la ingestión accidental por personas sensibles. Los niveles esperados son comparables con los encontrados en fórmulas infantiles hipoalergénicas.

Diversos autores han estudiado el efecto del SLP activado, aplicado de forma individual o combinado con otros tratamientos, sobre bacterias patógenas en distintas matrices alimentarias, como leche y productos lácteos (Zapico *et al.*, 1998; Arqués *et al.*, 2008), salmón ahumado (Montiel *et al.*, 2012). Sin embargo, pocos son los estudios publicados hasta la fecha sobre la aplicación del sistema SLP en carne o productos cárnicos (Wolfson *et al.*, 1994; Elliot *et al.*, 2004; Kennedy *et al.*, 2000).

1.2.2.3. Lactoferrina

La lactoferrina (LF) es una glicoproteína de aproximadamente 80 kDa de peso molecular que pertenece a la familia de las transferrinas. Está organizada en una estructura bilobular con capacidad para unir un átomo de hierro a cada uno de los lóbulos, a la vez que une un ión carbonato (Baker, 1994). Posee una alta afinidad por el hierro, siendo capaz de quelar hierro II y III de forma reversible, impidiendo el uso del mismo por los microorganismos y limitando su capacidad para multiplicarse. Existen tres formas de LF en función de la saturación de hierro que presenten: apolactoferrina (hierro libre), forma monoférrica (une un ión Fe^{+3}) y hololactoferrina (une dos iones Fe^{+3}). La capacidad de unir hierro, incluso a pH ácido es importante, especialmente en los sitios de infección e inflamación, donde debido al metabolismo bacteriano, el pH puede descender hasta 4.5, previniendo así su uso por parte de los microorganismos y evitando su proliferación (Valenti y Antonini, 2005). Se considera que su función fisiológica es el transporte de hierro, formando además parte del sistema inmune innato. Entre las funciones atribuidas a la LF se encuentra un posible papel como mediador en la absorción y transporte de hierro a nivel intestinal, dependiendo de las necesidades de hierro del organismo (Suzuki *et al.*, 2005). Se le atribuye además un potente efecto antimicrobiano, microbiostático y microbicida, frente a virus, bacterias, mohos, levaduras y protozoos, afectando a su capacidad de crecimiento y proliferación (Jenssen y Hancock, 2009). El efecto bacteriostático de la LF parece estar relacionado con su capacidad de secuestrar hierro, mientras que su actividad bactericida parece debida a su unión con los componentes de la pared celular o alteración de los mismos, tales como moléculas de lipopolisacáridos en bacterias Gram-negativas y ácidos teicoicos o lipoteicoicos en Gram-positivas. Como consecuencia, se produce despolarización, pérdida de la integridad de la membrana y del gradiente de pH (Vorland *et al.*, 1999). La LF posee derivados tales como su forma amidada (AMILF) y sus formas purificada (lactoferricina, LFC) y no purificada (PDLF), digeridas con pepsina. Debido a sus propiedades antibacterianas de amplio espectro, han sido propuestas como conservantes naturales en los alimentos (Naidu, 2002). En el mercado existe un producto comercial de la LF bovina, la LF activada-activin® (ALF) (ALF Ventures, LLC, Utah, EE.UU.) patentada en EE.UU. desde el año 2001, empleado para la higienización de canales mediante aplicación electrostática. Sin embargo, en la aplicación en alimentos, factores como la actividad de agua, pH, proteínas, lípidos, carbohidratos, y cationes, podrían interferir con su actividad antimicrobiana (Al-Nabulsi y Holley, 2007).

1.2.3. APH Y BIOCONSERVACIÓN EN PRODUCTOS CÁRNICOS

Entre los productos alimentarios procesados por APH, los productos cárnicos comercializados presurizados se han incrementado considerablemente (Garriga y Aymerich, 2009). El objetivo que persigue la industria cárnica es aumentar la seguridad de estos productos, inactivando microorganismos patógenos (principalmente *Listeria*), a la vez que se minimiza la alteración de los atributos que contribuyen a su calidad sensorial.

Diversas empresas ubicadas tanto en EE.UU. y Canadá como en países de la Unión Europea (Países Bajos, Rumanía, Grecia, Reino Unido y España), comercializan productos cárnicos presurizados (Bajovic *et al.*, 2012). En Europa, España fue pionera en la aplicación de la tecnología de APH en productos cárnicos, comercializando en 1998 jamón cocido loncheado presurizado a 400 MPa durante 10 min, que alcanzó una vida útil de 60 días a 4 ºC (Grebol, 2002). En la actualidad, productos como el jamón curado loncheado, chorizo, productos a base de pollo y pavo, mortadela, salami e incluso steak tartar y carpaccio, se encuentran presentes en el mercado de los productos cárnicos presurizados.

En la revisión llevada a cabo por Bajovic *et al.* (2012) se recogen los resultados obtenidos en diversas investigaciones con productos cárnicos sometidos a tratamientos de APH, aplicados de forma individual o en combinación con antimicrobianos. En este contexto, tratamientos de 400 y 600 MPa aplicados durante 10 min a 22 ºC en jamón cocido envasado a vacío, y almacenado en refrigeración durante 90 días, inhibieron la principal microbiota alterante del producto, aunque *Weissella viridiscens* y *Leuconostoc mesenteroides* sobrevivieron al tratamiento de presurización más intenso, siendo responsables de la alteración final del producto (Han *et al.*, 2011). Los tratamientos de 600 MPa durante 6 min a 31 ºC fueron efectivos en la inactivación de *L. monocytogenes*, *S. enterica*, *S. aureus*, *Y. enterocolitica*, *C. jejuni*, *E. coli* y la levadura *Debaryomyces hansenii* inoculados en loncheados de jamón cocido, jamón curado y lomo de ternera marinado, manteniéndose por debajo del límite de detección durante 120 días a 4 ºC (Jofré *et al.*, 2009). Por otro lado, Morales *et al*. (2006) observaron que la presurización a 450 MPa durante 10 min a 12 ºC de jamón Serrano e Ibérico loncheados, reducía los niveles de *L. monocytogenes* en 1.50 y 1.16 unidades logarítmicas, respectivamente, y aceleraba la muerte del patógeno durante el almacenamiento en condiciones de refrigeración.

Entre los escasos trabajos publicados de la aplicación de tratamientos de APH en carpaccio, Realini *et al*. (2011) observaron que tratamientos de 400 y 600 MPa

durante 6 min aplicados sobre carpaccio de cerdo, previamente congelado (-15 ºC y -35 ºC), minimizaba los cambios en los atributos de calidad del producto, siendo la temperatura inferior la más efectiva. Comprobaron que la presurización retrasaba el crecimiento de la microbiota alterante, aumentando la vida útil del carpaccio. Por otro lado, Szerman *et al.* (2011) observaron que la congelación del carpaccio de ternera y posterior presurización minimizaba los cambios de color y la capacidad de retención de agua se veía afectada en menor grado, al igual que la desnaturalización de las proteínas miofibrilares y sarcoplásmicas. Sin embargo, la inactivación microbiana alcanzada era menor en el carpaccio de ternera congelado presurizado, en comparación con las muestras descongeladas. Franceschini *et al.* (2005) detectaron que el tratamiento de 600 MPa durante 10 min, afectaba negativamente al color del carpaccio de ternera con una disminución en la tendencia al rojo (a^*).

En cuanto a la aplicación del SLP-GOX en productos cárnicos, Kennedy *et al*. (2000) observaron una gran variabilidad en la capacidad de crecimiento de los patógenos estudiados en carne picada, influida por las características de la cepa, la carga del inóculo y la temperatura, siendo mínima a temperaturas que permitan el crecimiento. En carne fresca de ternera, Elliot *et al*. (2004) detectaron que la actividad antimicrobiana del SLP-GOX, cuando la LPX era añadida a 1.9 U/cm^2 era bacteriostática, previniendo el crecimiento de bacterias patógenas a 12 ºC y reduciéndolo a temperaturas de enfriamiento.

En productos cárnicos se han realizado algunos estudios sobre la actividad antimicrobiana de la LF y sus derivados, observándose una disminución o pérdida de actividad en carne de ternera picada inoculada con *P. fluorescens* que podría ser atribuida a la presencia de compuestos de bajo peso molecular en la carne, como cationes (Del Olmo *et al.,* 2009). Por otro lado, el efecto bactericida de la ALF detectado en filetes de pollo inoculados con *L. monocytogenes* (Del Olmo *et al.,* 2012a) y *E. coli* O157:H7 (Del Olmo *et al.,* 2012b) fue muy leve.

Existen algunos trabajos publicados sobre la aplicación de bacteriocinas en productos cárnicos. Entre ellos, Hugas *et al*. (2002) observaron que la adición directa en salchichas tipo frankfurt de enterocina A y sakacina K reducía significativamente los niveles de *Listeria*. Tu y Mustapha (2002) detectaron que la combinación de EDTA y nisina añadidos sobre carne de ternera refrigerada envasada al vacío era efectiva en la inactivación de *B. thermosphacta*, consiguiendo aumentar la vida útil de la carne.

En cuanto a la aplicación de tratamientos combinados de APH y diferentes antimicrobianos en productos cárnicos, Hereu *et al*. (2012) observaron una mayor resistencia de *L. monocytogenes* inoculada en jamón curado loncheado con menor a_w

tratado a 600 MPa durante 5 min a 15 ºC. La combinación de las APH con nisina aplicada sobre la superficie de las muestras fue el tratamiento más efectivo. Aymerich *et al*. (2005) estudiaron la combinación de APH con nisina y sales de lactato para inhibir el crecimiento de *Salmonella* y *L. monocytogenes* en jamón cocido loncheado, observando que las células microbianas que habían sido dañadas subletalmente con la presurización resultaban sensibles a la nisina. Por otro lado, Garriga *et al*. (2002) estudiaron el comportamiento de varios patógenos inoculados en jamón cocido al que añadieron distintas bacteriocinas (enterocinas A y B, sakacina K, pediocina AcH y nisina), presurizaron a 400 MPa durante 10 min a 17 ºC y mantuvieron a 4 ºC durante 61 días. Observaron reducciones superiores a 6 unidades logarítmicas de *E. coli* en las muestras presurizadas combinadas con nisina y estos tratamientos mantuvieron a las BAL por debajo del límite de detección. La combinación de las APH con el resto de bacteriocinas mantuvo a *L. monocytogenes* por debajo del límite de detección y los niveles de *Salmonella* alcanzados después de la presurización permanecieron sin cambios durante el almacenamiento.

La actividad antimicrobiana de la ALF en carne de pollo inoculada con *L. monocytogenes* (Del Olmo *et al.*, 2012a) y *E. coli* O157:H7 (Del Olmo *et al.*, 2012b) en combinación con APH a 400 MPa durante 10 min a 10 ºC, mejoró ligeramente la inactivación de ambos patógenos respecto a la aplicación individual de los antimicrobianos.

1.3. OBJETIVOS DE LA TESIS

Los objetivos de la presente tesis doctoral se centran en conseguir la máxima seguridad microbiológica de productos cárnicos listos para el consumo (RTE) mediante tratamientos combinados de altas presiones y bioconservación.

En este contexto, se plantearon los siguientes objetivos parciales:

1.- Inactivar a los patógenos *L. monocytogenes*, *S. enterica* ser. Enteritidis y *E. coli* O157:H7 en jamón curado loncheado y en carpaccio de ternera mediante tratamientos de alta presión aplicados individualmente o en combinación con bacteriocinas, el sistema lactoperoxidasa o lactoferrina.

2.- Evaluar los cambios en las características fisicoquímicas, reológicas y en el color de los productos como consecuencia de los tratamientos de inactivación.

3.- Investigar *in vitro* nuevos tratamientos combinados de nitrito sódico y altas presiones para la inactivación de *L. monocytogenes* y *E. coli*.

2. Materiales y métodos

2. MATERIALES Y MÉTODOS

2.1. MICROORGANISMOS

A continuación se indican los microorganismos utilizados en los experimentos realizados en la presente tesis doctoral:

ESPECIE	CEPA	ORIGEN
Listeria monocytogenes	INIA H66a	Colección cultivos INIA (Dpto. Tecnología de Alimentos)
	CECT 4032	Universidad de Reading (Reino Unido)
Salmonella enterica	CECT 4155	Colección Española de Cultivos Tipo
	CECT 4300	Colección Española de Cultivos Tipo
	CECT 4396	Colección Española de Cultivos Tipo
Escherichia coli	CECT 4972	Colección Española de Cultivos Tipo
	BW25113	Universidad de Reading (Reino Unido)
	BW25113 *hmp*A	Universidad de Reading (Reino Unido)
	BW25113 *nor*V	Universidad de Reading (Reino Unido)
Lactococcus lactis	INIA TAB 26	Colección cultivos INIA (Dpto. Tecnología de Alimentos)
Enterococcus faecium	INIA TAB 7	Colección cultivos INIA (Dpto. Tecnología de Alimentos)
Pediococcus acidilaciti	347	Universidad Complutense de Madrid
Listeria innocua	BL86/26	Università Cattolica del Sacro Cuore (Italia)
Lactobacillus buchneri	St2A	NIZO Food Research (Holanda)

2.2. TRATAMIENTOS DE INACTIVACIÓN INVESTIGADOS

Los tratamientos de inactivación investigados, descritos a continuación, se aplicaron de forma individual o combinados con altas presiones hidrostáticas:

2.2.1. TRATAMIENTOS DE BIOCONSERVACIÓN

2.2.1.1. Preparados comerciales de bacterias lácticas y bacteriocinas

Se han ensayado un fermentado de un cultivo iniciador de *Pediococcus acidilactici* productor de pediocina (Fargo, Laboratorios Amerex, Madrid, España) y un preparado comercial de nisina con una pureza del 2.5 % (Sigma-Aldrich, Alcobendas, España).

2.2.1.2. Sobrenadantes de bacterias lácticas

Se utilizaron sobrenadantes de cultivos de *L. lactis* INIA TAB 26, *E. faecium* INIA TAB 7 y *P. acidilactici* 347 productores de nisina Z, enterocinas A y B y pediocina PA-1, respectivamente.

2.2.1.3. Sistema lactoperoxidasa

El sistema lactoperoxidasa utilizado estaba constituido por la enzima lactoperoxidasa de leche bovina (DMV International, Barcelona, España), que se activó mediante

tiocianato potásico (Merck, Darmstadt, Alemania), glucosa (Panreac, Barcelona, España) y glucosa oxidasa (Sigma-Aldrich).

2.2.1.4. Lactoferrina

Se empleó lactoferrina bovina parcialmente saturada de hierro (15-20%) según el fabricante (DMV International), y lactoferrina activada en su forma comercial (LF activada-activin^{R}).

2.2.2. ALTAS PRESIONES HIDROSTÁTICAS (APH)

Los tratamientos de presurización que se aplicaron a las muestras de jamón curado loncheado y a las muestras de carpaccio de ternera se llevaron a cabo en un equipo de altas presiones ACIP 6000 (ACB, Nantes, Francia) de 3.5 L de capacidad que alcanza como máximo 600 MPa de presión. El fluido transmisor fue el agua.

Por otro lado, para aplicar los tratamientos de presurización a los cultivos de *E. coli* BW25113, y sus mutantes *hmp*A, *nor*V y de *L. monocytogenes* CECT 4032 realizados en la Universidad de Reading se empleó un equipo de altas presiones de 300 mL de capacidad (modelo S-FL-850-9-W, Stansted, Reino Unido). Se utilizó monopropilen glicol-agua (30:70) como fluido transmisor de la presión.

2.3. METODOLOGÍA

2.3.1. CONDICIONES DE CULTIVO DE LOS MICROORGANISMOS

Las cepas de *S*. Enteritidis, *L. monocytogenes* INIA H66a, *E. coli* O157:H7 y *L. innocua* se conservaron a -80 ºC en caldo tripticasa soja con extracto de levadura (TSYEB, Biolife, Milán, Italia) suplementado con 30% de glicerol. Con el objetivo de alcanzar la fase estacionaria y ser utilizadas en los experimentos planteados, se sembraron en caldo TSYEB e incubaron a 37 ºC durante 18 h.

L. lactis, *E. faecium*, *P. acidilactici* y *L. buchneri* se conservaron a -80 ºC en caldo Man Rogosa y Sharpe (MRS, Biolife) suplementado con 30% de glicerol. Con el objetivo de alcanzar la fase estacionaria y ser utilizadas en los experimentos planteados, fueron cultivadas en caldo MRS e incubadas a 30 ºC durante 18 h.

Las cepas de *L. monocytogenes* CECT 4032 y *E. coli* BW25113, *hmp*A y *nor*V, se conservaron a -70 ºC en una solución hipertónica de crioconservación (Cryobeads, Neston, Reino Unido). Para ser utilizadas en los experimentos correspondientes, fueron crecidas en caldo TSB (Biolife) a 37 ºC durante 6 h y tras realizar una dilución 1:1000 en caldo TSB, se incubaron a 37 ºC hasta alcanzar la fase estacionaria.

2.3.2. PREPARACIÓN DE LAS MUESTRAS

Se prepararon muestras de jamón curado loncheado de 20 g y muestras de carpaccio de ternera de 10 g para ser inoculadas con los microorganismos patógenos. Por otro lado, se prepararon muestras sin inocular para realizar el recuento de bacterias aerobias totales en ambas matrices alimentarias, y el resto de determinaciones planteadas a continuación.

Las determinaciones de pH y a_w se efectuaron en muestras de 10 g de jamón curado loncheado y en muestras de 5 g de carpaccio de ternera. Para el análisis de aminoácidos libres y determinación del grado de oxidación lipídica, se emplearon muestras de 5 y 2.5 g de jamón curado picado libre de grasa, respectivamente.

Para llevar a cabo las determinaciones de color en jamón curado loncheado se prepararon muestras de 2 mm de grosor y para efectuar las determinaciones de textura se prepararon muestras de 4 mm de grosor. Respecto a las muestras de carpaccio de ternera, se prepararon con un espesor de 2 mm para ambas determinaciones.

Las muestras se depositaron en bolsas estériles BB325, con permeabilidad al oxígeno de 25 cm^3/m^2 (Cryovac Grace S.A., Barcelona, España) y se mantuvieron a 4 ºC hasta la aplicación de los tratamientos de presurización.

2.3.3. INOCULACIÓN DE LOS MICROORGANISMOS

Los microorganismos patógenos fueron inoculados en la superficie de las muestras para alcanzar un nivel de contaminación final de aproximadamente 10^6 ufc/g.

Por otro lado, se inocularon 10 mL de TSB con una colonia de *E. coli* BW25113, *hmp*A, *nor*V o *L. monocytogenes* CECT 4032 y se incubaron a 37 ºC durante 6 h. A continuación, y tras realizar una dilución 1:1000 en caldo TSB, los cultivos fueron incubados a 37 ºC hasta alcanzar el inicio de la fase estacionaria.

2.3.4. ADICIÓN DE BIOCONSERVANTES

2.3.4.1. Preparados comerciales de bacterias lácticas y bacteriocinas

El preparado comercial de *P. acidilactici* fue disuelto en agua destilada-desionizada estéril y se añadió a las muestras a una concentración final del 0.6%. La nisina comercial con una actividad de 10^6 UI/g, según el fabricante, se preparó en HCl 0.02 M (0.02 g/mL), obteniéndose una solución con una actividad de 20000 UI/mL. Se añadió a las muestras a una concentración final de 100 UI/g, equivalente a 100 µg de nisina /g producto. La adición de ambos bioconservantes se realizó en la superficie de

las muestras antes de la aplicación de los correspondientes tratamientos de presurización.

2.3.4.2. Sobrenadantes de bacterias lácticas productoras de bacteriocinas

Los cultivos de bacterias lácticas productoras de bacteriocinas previamente inoculados al 1% en caldo MRS e incubados durante 18 h a 30 ºC, fueron sometidos a centrifugación (10000 g durante 15 min a 4 ºC) en centrífuga Eppendorf 5810R (Eppendorf, Hamburgo, Alemania). Los sobrenadantes obtenidos fueron neutralizados a pH 6.0 con NaOH 1N y filtrados con filtros de acetato de celulosa de baja retención de proteínas de 0.22 µm de tamaño de poro (Millipore, Bedford, MA, EE.UU.). La actividad inhibitoria de los sobrenadantes neutralizados fue determinada mediante un test de difusión en pocillo, usando *L. innocua* BL86/26 y *L. buchneri* St2A como cepas indicadoras. Se prepararon placas de TSYEA y MRS agar con doble capa de agar. La segunda capa con un 0.7% de agar fue inoculada con el microorganismo indicador al 0.1%. Se depositaron volúmenes de 25 µL de sobrenadante en pocillos de 5 mm de diámetro y las placas fueron incubadas a 37 o 30 ºC durante 24 h. La presencia de halos de inhibición, que se correspondía con ausencia de crecimiento, revelaba actividad bacteriocina. Dicha actividad fue expresada en unidades arbitrarias (UA) y definida como el inverso de la máxima dilución que mostró actividad inhibitoria frente a las cepas indicadoras. Las bacteriocinas se añadieron a las muestras como sobrenadantes neutralizados y filtrados.

2.3.4.3. Sistema lactoperoxidasa

Se preparó lactoperoxidasa bovina (LP) a una concentración de 15 mg/mL en agua destilada-desionizada, se esterilizó con filtros de acetato de celulosa de 0.2 µm de tamaño de poro y almacenó a -40 ºC hasta su uso. El tiocianato potásico (KSCN) se preparó a una concentración de 1 M y fue esterilizado a 121 ºC durante 15 min. La glucosa y la glucosa oxidasa (GOX) se prepararon a concentraciones de 1 M y 2260 U/mL, respectivamente. Tras ser esterilizadas con filtros de acetato de celulosa, las soluciones se mantuvieron a temperatura ambiente y a 4 ºC hasta su uso, respectivamente.

La actividad LP fue cuantificada por triplicado antes de la adición al alimento, según el método de Marshall *et al*. (1986), consistente en la determinación de la oxidación del ABTS (2,2-azino-bis-(3-etilbenzotiazolin-ácido 6-sulfónico)) (Sigma-Aldrich) a 412 nm. Para ello, se preparó el blanco de la reacción compuesto por 2.95 mL de la solución de ABTS 1 mM en tampón acetato sódico 0.1M y 30 µL de una solución de LP preparada a una concentración de 15 µg/mL. A continuación, se añadieron 30 µL de H_2O_2 10 mM en tampón acetato. La oxidación del ABTS se determinó mediante los

valores de absorbancia obtenidos durante 5 min en un espectrofotómetro Beckman DU 650 (Beckman Instruments, Fullerton, EE.UU.). La actividad LP fue expresada en unidades de ABTS, según el método descrito por Shindler *et al*. (1976).

El sistema lactoperoxidasa fue activado *in vitro*, añadiendo la enzima con una actividad de 2.8 U ABTS/g, 0.03 mg/g de tiocianato potásico, 0.16 mg/g de glucosa y 0.15 U/g de GOX, e inmediatamente añadido a la superficie de las muestras, según recomendación de la FSANZ (2002).

2.3.4.4. Lactoferrina

La lactoferrina se preparó a una concentración de 40 mg/mL en agua destilada-desionizada, se filtró mediante filtros de acetato de celulosa de 0.22 µm de tamaño de poro y se almacenó a -40 ºC hasta su uso. La LF bovina se añadió a las muestras a una concentración de 1 mg/g.

La lactoferrina activada (ALF) se preparó a una concentración de 20 mg/mL en agua destilada-desionizada, se esterilizó con filtros de acetato de celulosa de 0.22 µm de tamaño de poro y se almacenó a -40 ºC hasta su uso. Las muestras fueron tratadas con ALF a una concentración de 1 mg/g.

2.3.5. TRATAMIENTOS DE ALTAS PRESIONES HIDROSTÁTICAS

Los tratamientos de presurización aplicados a las muestras de jamón curado loncheado y carpaccio de ternera, envasadas al vacío, y a temperatura de aproximadamente 12 ºC, fueron los siguientes:

- En jamón curado loncheado, las intensidades y duración de los tratamientos aplicados fueron de 400 MPa durante 5 y 10 min, 500 MPa durante 5 y 10 min, 600 MPa durante 5 min y 450 MPa durante 10 min.
- En carpaccio de ternera, los tratamientos aplicados fueron de 450 MPa durante 5, 10 y 15 min.

Los tratamientos de presurización que se aplicaron en cultivos, a 18 ºC de temperatura se describen a continuación:

- Tratamientos de 300 MPa durante 8 min frente a *E. coli* BW25113, *hmp*A *y nor*V.
- Tratamientos de 225 MPa durante 8 min frente a *L. monocytogenes* CECT 4032.

2.3.6. ANÁLISIS MICROBIOLÓGICO

2.3.6.1. Recuentos de *L. monocytogenes* INIA H66a, *S.* Enteritidis y *E. coli* O157:H7

Las muestras fueron homogenizadas con agua de peptona estéril al 0.1% en un homogenizador (IUL, Barcelona, España) durante 90 s. Se realizaron diluciones seriadas del homogenizado en la misma solución estéril y se sembraron placas duplicadas de Chromagar Listeria (CHROMagar, París, Francia) para el recuento de *Listeria*, que se incubaron a 37 ºC durante 48 h, Salmonella Shigella agar (SSA, Scharlab S.L., Barcelona, España) para *Salmonella* y agar biliado-rojo neutro-cristal Violeta (VRBA, Oxoid Ltd., Basingstoke, Inglaterra) para *E. coli* O157:H7, que fueron incubadas a 37 ºC durante 24 h. En los casos en los que no se detectó *Salmonella* por recuento directo, se investigó la presencia o ausencia del patógeno mediante enriquecimiento inicial en agua de peptona al 0.1%, enriquecimiento en caldo líquido selectivo Rappaport-Vassiliadis (RV, Oxoid Ltd., Basingstoke, UK) y siembra en estría en medio sólido selectivo SSA.

2.3.6.2. Recuentos de bacterias aerobias totales

Las muestras fueron homogenizadas como se indica en el apartado anterior y se sembraron placas duplicadas de TSA que se incubaron a 30 ºC durante 72 h para el recuento de bacterias aerobias totales.

2.3.6.3. Recuentos de *E. coli* BW25113, *hmp*A, *nor*V y *L. monocytogenes* CECT 4032

Los cultivos de *E. coli* BW25113, *hmp*A, *nor*V y *L. monocytogenes* CECT 4032 fueron centrifugados a 10000 g durante 5 min a 4 ºC y los pellets resuspendidos en tampón citrato-fosfato a pH 4.0. Se añadieron concentraciones de 0.25, 0.50 y 0.75 mM de nitrito sódico ($NaNO_2$) al tampón para ser ensayadas frente a *L. monocytogenes* CECT 4032 y concentraciones de 1.0, 2.0 y 3.0 mM de $NaNO_2$ para *E. coli* BW25113. Las suspensiones celulares fueron diluidas en el tampón adecuado hasta 10^8 ufc/mL y se dispensaron muestras de 1 mL en bolsas de plástico de polietileno de alta densidad estériles (2 cm x 5 cm y 65 µm de grosor; Seward Ltd., Worthing, West Sussex, Reino Unido). A continuación, se aplicaron tratamientos de APH con las intensidades descritas en el apartado 2.3.5. Por otra parte, se ensayaron distintos valores de pH (4.0, 5.0 y 7.0), $NaNO_2$ 2.0 mM y APH a 300 MPa durante 8 min, aplicadas de forma individual o combinada frente a *E. coli* BW25113, así como APH y $NaNO_2$ 3.0 mM frente a *E. coli* BW25113, *hmp*A y *nor*V a pH 4.0.

Para realizar los correspondientes recuentos de células viables, las muestras se diluyeron en agua de peptona salina (MRD, Oxoid) y se sembraron en placas

duplicadas de TSA suplementado con extracto de levadura (TSYEA) al 0.3% y piruvato sódico al 0.1%. Las placas se incubaron a 37 ºC durante 24 y 48 h para el recuento de *E. coli* y *Listeria*, respectivamente.

2.3.7. ANÁLISIS QUÍMICOS

2.3.7.1. Medida de pH

Los valores de pH se midieron por duplicado en homogenizados de las muestras en agua destilada estéril, empleando un pH-metro (modelo GPL22, Crison Instruments, Barcelona, España).

2.3.7.2. Medida de actividad de agua (a_w)

Los valores de a_w de las muestras se determinaron por duplicado con un equipo AquaLab Series 3 (Decagon Devices, Inc., Pullman, WA, EE.UU.), con un sensor de punto de rocío.

2.3.7.3. Determinación de la oxidación de lípidos o índice TBA-RS

El grado de oxidación lipídica en jamón curado se determinó como índice TBA-RS (sustancias reactivas con el ácido tiobarbitúrico), según Pikul *et al*. (1989). Se pesaron muestras de 2.5 g de jamón picado, libre de grasa, y se homogenizaron con 20 mL de agua MilliQ. Se añadieron 5 mL de ácido tricloroacético (TCA) y los homogenizados se centrifugaron a 10000 g durante 15 min a 4 ºC. Los sobrenadantes obtenidos se filtraron con filtros Whatman nº 54 (Whatman™, Buckinghamshire, Reino Unido) y se añadieron a 3.5 mL del filtrado, volúmenes de 1.5 mL de ácido tiobarbitúrico (TBA) al 0.6%. La mezcla se incubó a 70 ºC durante 30 min en un baño de agua con agitación. A continuación, las muestras se enfriaron en hielo durante 10 min y se mantuvieron a temperatura ambiente durante 2 horas. Se midió la absorbancia a 532 nm. Los resultados se expresaron como mg de malonaldehído (MDA) por kg de muestra (Pryor *et al.,* 1976), utilizando una recta patrón.

2.3.7.4. Determinación de aminoácidos libres

La extracción de aminoácidos libres se realizó en muestras de jamón loncheado picado, libre de grasa, según el método descrito por Aristoy *et al*. (1991) con algunas modificaciones. Se homogenizaron 5 g de muestra picada libre de grasa con 375 µL de patrón interno (655.9 mg de L-Norleucina/30 mL de HCl 0.1 M) y 25 mL de HCl 0.1 M durante 8 min en un homogenizador. Se centrifugó a 10000 g durante 20 min a 4 ºC y los sobrenadantes obtenidos fueron filtrados con lana de vidrio y filtro de membrana de 0.45 µm de tamaño de poro (Millipore). Se desproteinizaron

volúmenes de 400 µL de cada filtrado mediante filtros Ultra Free MC (Millipore) con masa molecular nominal de 5000 Da (previamente lavados con 200 µL de HCl 0.1 M) y se centrifugaron a 4500 g durante 60 min a 2-4 ºC. Los aminoácidos fueron derivatizados con 6-aminoquinolil-N-hidroxisuccinimidil carbamato, usando el Kit Reactivo AccQ-Fluor (Waters Corporation, Milford, MA, EE.UU.). Los aminoácidos libres se determinaron por HPLC en fase reversa, siguiendo las instrucciones del fabricante, en una columna Waters AccQ-Tag de análisis de aminoácidos en un cromatógrafo Beckman System Gold (Beckman Instruments) equipado con un detector de fluorescencia usando una longitud de onda de excitación de 250 nm y de emisión de 395 nm. Los aminoácidos fueron identificados y cuantificados inyectando concentraciones conocidas de aminoácidos puros (SIGMA). Los resultados se expresaron como mg de aminoácidos libres por 100 g de jamón curado.

2.3.8. ANÁLISIS DE COLOR

El color de las muestras de jamón y carpaccio se determinó con un colorímetro Minolta CM 700d (Minolta Camera Co., Osaka, Japón). Se midieron la luminosidad (L^*), la tendencia al rojo (a^*) y la tendencia al amarillo (b^*). Se realizaron seis medidas por músculo por duplicado en el caso del jamón curado y seis determinaciones por muestra en el carpaccio también por duplicado.

2.3.9. ANÁLISIS REOLÓGICO

Los determinaciones de textura, fuerza al corte (N) y fuerza máxima (N), fueron medidas con una prensa Instron Compression Tester 4301 (Instron Ltd., Barcelona, España) controlado por un software BlueHill V2.0, con una célula de carga de 1000 N y velocidad de 100 mm/min. La fuerza al corte se determinó con una celda Warner-Bratzler y la fuerza máxima con una celda Kramer. Se realizaron tres determinaciones de cada parámetro por músculo por duplicado en el jamón curado y seis determinaciones en carpaccio.

2.3.10. ANÁLISIS ESTADÍSTICO

Para el análisis estadístico de los datos obtenidos experimentalmente se realizó un análisis de varianza mediante el programa SPSS Win 12.0 (SPSS Inc., Chicago, IL) considerando el tratamiento y el tiempo de almacenamiento como efectos principales. Las diferencias significativas entre medias fueron evaluadas mediante el Test de Tukey con un intervalo de confianza del 95%.

3. Resultados

3.1. High pressure treatments on the inactivation of *Salmonella* Enteritidis and the physicochemical, rheological and color characteristics of sliced vacuum-packaged dry-cured ham. *Meat Science (2012) 91:173-178.*

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High pressure treatments on the inactivation of *Salmonella* Enteritidis and the physicochemical, rheological and color characteristics of sliced vacuum-packaged dry-cured ham

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ABSTRACT

The effect of high pressure (HP) on *Salmonella* Enteritidis in sliced dry-cured ham stored under temperature abuse (8 °C) during 60 d was investigated. After treatment, reductions of *S.* Enteritidis were 1.06, 2.54 and 4.32 log units in ham treated at 400, 500 and 600 MPa for 5 min at 12 °C, compared to non-pressurized samples. After 60 d, counts of *S.* Enteritidis in ham treated at 400 and 500 MPa were 2.56 and 2.66 log units lower than in non-treated ham, whereas the pathogen was only detected after enrichment in ham treated at 600 MPa. Lipid oxidation increased with storage and pressurization, whereas total free amino acid contents were similar in HP and control samples after 60 d. Dry-cured ham treated at the highest pressures exhibited lower shear resistance, whereas the maximum force to compress the sample was slightly changed. Color (L^*, a^* and b^*) varied with pressurization and storage. Changes induced by HP in dry-cured ham were attenuated during storage.

1. Introduction

Salmonella has long been recognized as an important zoonotic pathogen of economic significance in animals and humans. Although *Salmonella* is a ubiquitous organism, the primary reservoir of *Salmonella* is the intestinal tract of a wide range of animals which result in a variety of foodstuffs covering both food of animal and plant origin as sources of infection. Transmission often occurs when the pathogen is introduced in food preparation areas and allowed to multiply in food, e.g. due to inadequate storage temperatures, inadequate cooking or cross contamination of ready-to-eat (RTE) foods. In total 108,614 confirmed human cases were reported in the European Union in 2009, although cases caused by *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) decreased markedly (EFSA, 2011). On the contrary, *Salmonella* infections have not declined over the past 15 years in the United States (CDC, 2011).

Bacteria of public health concern are not generally associated with dry-cured ham (Reynolds, Harrison, Rose-Morrow, & Lyon, 2001) due to its low water activity (a_w) and high salt and nitrite levels. However, sliced dry-cured ham is a RTE product which can be contaminated by exposure to the environment during slicing and packaging operations. Although considered a product with a long shelf-life at refrigeration temperature, the risk of spoilage or food-pathogen growth increases in the case of temperature abuse conditions over a long storage period (Cava, Ladero, Gonzalez, Carrasco, & Ramirez 2009).

High pressure (HP) treatment is a non-thermal method of food preservation used for a wide range of products to ensure their microbiological safety and extend their shelf-life while maintaining quality. The pressure applied (200–1000 MPa) is instantaneously and uniformly transmitted independent of the size and geometry of the food. Industrial HP applications have increased rapidly, especially in the stabilization of RTE meats and cured products. HP has been recommended by regulatory agencies (FDA, US Food and Drug Administration) as an effective post-packaging technology to control *Listeria monocytogenes* mainly in RTE products. Changes in color, texture and water-holding capacity by HP treatments in meat, meat products and seafood have been reviewed by Campus (2010). The effect of pressurization on sliced dry-cured ham characteristics has been focused on lipid and protein oxidation, which was enhanced, and on color changes, with lightness reported to increase and redness decrease (Andrés, Moller, Adamsen, & Skibsted, 2004; Andrés, Adamsen, Moller, Ruiz, & Skibsted, 2006; Cava et al., 2009; Fuentes, Ventanas, Morcuende, Estévez, & Ventanas, 2010).

Microorganisms are adversely affected by HP treatment through damage to the cell wall, membranes, enzymes and nucleic acids (Hoover, Metrick, Papineau, Farkas, & Knorr, 1989; Smelt, 1998). The inactivation of *Salmonella* by HP treatments has been investigated in different food substrates such as liquid whole egg, minced chicken, milk, cheese, low-acid fermented sausages, RTE meat products and chicken breast fillets (De Lamo-Castellví, Roig-Sagués, López-Pedemonte, Hernández-Herrero, Guamis, & Capellas, 2007; Guan, Chen, & Hoover,

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2005; Jofré, Aymerich, Monfort, & Garriga, 2008b, Jofré, Garriga, & Aymerich, 2008a; Marcos, Aymerich, & Garriga, 2005; Morales, Calzada, Rodríguez, De Paz, & Nuñez, 2009; Ponce, Pla, Sendra, Guamis, & Mor-Mur 1999; Yuste et al., 2003). The effect of pressurization on the spoilage microbiota of dry-cured ham was investigated by Garriga, Grebol, Aymerich, Monfort, and Hugas (2004), who concluded that HP at 600 MPa for 6 min prevented the growth of *Enterobacteriaceae* and yeasts and delayed the growth of lactic acid bacteria. The inactivation of *L. monocytogenes* in pressurized Iberian and Serrano sliced cured hams (Morales, Calzada, & Nuñez, 2006) and the efficacy of bacteriocins in combination with HP at 600 MPa against *S. enterica*, *Staphylococcus aureus* and *L. monocytogenes* in dry-cured ham (Jofré et al., 2008b) have been reported.

The aim of this study was to evaluate the effect of high pressure treatments on the inactivation of inoculated *S. enterica* subsp. *enterica* serovar Enteritidis, together with the physicochemical, rheological and color characteristics of sliced vacuum-packaged dry-cured ham stored under temperature abuse conditions (8 °C) during 60 d.

2. Material and methods

2.1. Microorganisms

S. Enteritidis strains CECT 4155, CECT 4300, and CECT 4396 from the Spanish Type Culture Collection (Valencia, Spain) were kept frozen at −80 °C in tryptic soy yeast extract broth (TSYEB) (Biolife, Milano, Italy) with 10% glycerol. A three-strain cocktail of *S.* Enteritidis was prepared by mixing equal amounts of cultures of *Salmonella* strains, separately grown in TSYEB for 18 h at 37 °C.

2.2. Sample preparation and HP treatments

Sliced dry-cured Serrano ham samples (20 g) were inoculated by spreading 100 μl of the cocktail of *Salmonella* strains on the surface in order to achieve a final population of approximately 10^6 cfu/g. Inoculated slices were individually vacuum-packed in double bags of BB325 (Cryovac Grace S.A., Barcelona, Spain) and held at 4 °C for 20 h before pressurization. Non inoculated samples were also prepared to measure pH, a_w, lipid oxidation, content of free amino acids (FAA), texture and color. Dry-cured ham was sliced into 4-mm-thick slices to measure texture and 2-mm-thick slices to measure color.

High pressure treatments were performed in a high-pressure batch apparatus (model ACIP 6000; ACB, Nantes, France), of 3.5-l capacity and 600 MPa maximum working pressure. Samples were pressurized at three levels (400 MPa, 500 MPa and 600 MPa) and one holding time (5 min) at approx. 12 °C giving three experimental treatments (400 MPa, 500 MPa and 600 MPa during 5 min, respectively), as well as a non-pressurized control group. Pressure come up time ranged between 2 and 4 min and depressurization time was 25 s. After pressure treatment, pressurized and non-pressurized samples were stored at 8 °C for 60 d. Two independent trials were carried out.

2.3. Microbiological analysis

Samples were analyzed for *Salmonella* at 8 h, and 1, 3, 7, 15, 30, 45 and 60 d after HP treatments. Samples (20 g) were homogenized in 180 ml of 0.1% sterile peptone water using a homogenizer (IUL, Barcelona, Spain). Decimal dilutions of the homogenate were prepared in the same sterile solution. *Salmonella* population was determined on duplicate plates of Salmonella Shigella agar (SSA; Scharlab S.L., Barcelona, Spain) incubated at 37 °C for 24 h. If necessary, an initial enrichment step was included by adding the samples (20 g) to 180 ml of 0.1% peptone water and incubating at 37 °C for 24 h. This was followed by a secondary enrichment where 100 μl of the primary enrichment broth was added to 9 ml Rappaport-Vassiliadis broth (Oxoid Ltd., Basingstoke, England). After incubation at 37 °C for 24 h, enrichment broth was streaked on duplicate plates of SSA which were examined after incubation at 37 °C for 24 h to detect presence or absence of *Salmonella*.

2.4. pH and a_w

pH determinations were taken using a pH-meter (model GPL22, Crison Instruments, Barcelona, Spain). Samples (10 g) of dry-cured ham were homogenized in 90 ml of distilled water. Water activity was measured in *Quadriceps femoris* and *Biceps femoris* muscles using the AquaLab Series 3 equipment (Decagon Devices, Inc., Pullman, WA, USA). Samples were taken 1, 15, 30, 45 and 60 d after treatment. All measurements were made in duplicate samples.

2.5. Lipid oxidation

The extent of lipid oxidation was estimated as TBARS (Thiobarbituric acid reactive substances) as described by Pikul, Leszczynski, and Kummerow (1989). Samples (2.5 g) of minced ham without fat were homogenized in 20 ml of MilliQ water, 5 ml of TCA were added and samples were centrifuged at 10,000 *g* for 15 min at 4 °C. The supernatants were filtered through Whatman filter number 54 and 1.5 ml of 0.6% TBA was added to 3.5 ml of filtrate. The mixture was incubated at 70 °C for 30 min in a water bath with agitation. Samples were cooled in ice for 10 min and left at room temperature for 2 h. Absorbance was measured at 532 nm in a Beckman DU 650 spectrophotometer (Beckman Instruments SA, Madrid, Spain). The results were expressed as mg malondialdehyde (MDA)/kg sample (Pryor, Stanley, & Blair, 1976). Analyses were carried out 1, 30 and 60 d after treatment.

2.6. Free amino acid (FAA) analysis

FAA were extracted from dry-cured minced ham according to Aristoy and Toldrá (1991). In brief, 5 g of sample without fat was homogenized with 375 μl of internal standard (655.9 mg of L-Norleucine/30 ml of 0.1 M HCl) and 25 ml of 0.1 M HCl in a homogenizer for 8 min and centrifuged at 10,000 *g* for 20 min at 5 °C. The supernatants were filtered through glass wool and a membrane filter (0.45 μm pore dimension) and 400 μl of each filtrate was deproteinized using Ultra Free MC filters (Millipore Iberica SAU, Madrid, Spain) with nominal molecular mass cut-off of 5000 Da (before washing with 200 μl of 0.1 M HCl) and centrifuged at 4500 *g* for 60 min at 2–4 °C. The amino acids were derivatized using with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate using the AccQ-Fluor Reagent Kit (Waters Corporation, Milford, MA, USA) and individual amino acids determined by RP-HPLC in a Waters AccQ·Tag Amino Acid Analysis column using a Beckman System Gold chromatograph (Beckman Instruments) equipped with a fluorescence detector with excitation wavelength at 250 nm and emission wavelength at 395 nm. The amino acids were identified and quantified using standard curves. Results were expressed as mg per 100 g of dry-cured ham 1, 30 and 60 d after treatment.

2.7. Textural determinations

Texture parameters were measured using an Instron Compression Tester 4301 (Instron Ltd., Barcelona, Spain) with a load cell of 1000 N and crosshead speed of 100 mm/min. The shear force (N) was determined with a Warner-Bratzler blade and the maximum force (N) required to compress the sample with a Kramer cell. Three measurements were taken at each muscle (*Q. femoris* and *B. femoris*) in dry-cured ham 1, 15, 30, 45 and 60 d after treatment.

2.8. Color measurements

Surface color of sliced dry-cured ham was measured six times on each muscle (*Q. femoris* and *B. femoris*) using a Minolta CM 700d spectrophotometer (Minolta Camera Co., Osaka, Japan). L^* (lightness), a^* (redness) and b^* (yellowness) were determined 1, 15, 30, 45 and 60 d after treatment.

2.9. Statistical analysis

Data were subjected to analysis of variance (ANOVA) by means of SPSS program Win 12.0 software (SPSS Inc., Chicago, IL), with treatment and time of storage as main effects. Significant differences between means were assessed by Tukey's test at $P<0.05$.

3. Results and discussion

3.1. Inactivation of Salmonella Enteritidis

Counts of *S.* Enteritidis in sliced vacuum packaged dry-cured ham subjected to high pressure treatments at 400 MPa, 500 MPa and 600 MPa during 5 min and stored at 8 °C are shown in Table 1. According to the analysis of variance, high pressure treatment and storage time had significant ($P<0.001$) effects on *Salmonella* counts. *S.* Enteritidis mean initial counts in dry-cured ham immediately after inoculation were 6.30 log cfu/g and after 20 h at 4 °C were 6.20 log cfu/g. The population of *Salmonella* declined by 2.31 log cfu/g in non-pressurized ham during 60 d of storage even under the temperature abuse conditions (8 °C). Lethality values increased with the level of pressurization. In dry-cured ham 8 h after pressurization, SSA counts in 400, 500 and 600 MPa treated samples were 1.06, 2.54 and 4.32 log cfu/g lower than in non-pressurized dry-cured ham. A gradual decline was observed throughout refrigeration, particularly at higher pressures. Differences increased after 7 d, with counts in 400 and 500 MPa pressurized samples being 1.46 and 3.00 log cfu/g lower than in non-pressurized dry-cured ham, whereas *Salmonella* was only detected after enrichment of samples treated at 600 MPa (presence in 20 g; >5 log reductions). At the end of the refrigeration period, counts of *S.* Enteritidis were 2.56 and 2.66 log cfu/g lower in dry-cured ham treated at 400 and 500 MPa, respectively, than in non-pressurized samples and the pathogen counts were below the detection limit but their presence (in 20 g) was recorded in samples treated at 600 MPa.

Damaged cells were not able to repair during 60 d of storage at 8 °C in the dry-cured ham, showing a decrease in their counts on selective SSA. This decrease was observed also in non-pressurized dry-cured ham, indicating that cells were affected by the adverse conditions of this meat product, although HP treatments accelerated *Salmonella* inactivation. Repair of food-borne pathogens during storage is important for HP treated foods because safety can be overestimated during refrigerated storage. Sublethally damaged cells might recover depending on the strain, the species and the environmental conditions, mainly the physicochemical properties of the food and the storage temperature (Jofré, Aymerich, Bover-Cid, & Garriga, 2010). Stressed cells may recover better under anaerobic conditions, such as vacuum packaging. However in the case of dry-cured ham, HP induced injured cells could not repair under the unfavorable conditions of low a_w and high NaCl in the product.

Enterobacteriaceae did not grow in HP treated dry-cured ham at 600 MPa during 6 min and the growth of lactic acid bacteria was delayed (López-Caballero, Carballo, & Jiménez-Colmenero, 1999). The population of *Salmonella* in cooked ham pressurized at 600 MPa for 5 min was reduced from 4 log cfu/g to levels below 10 cfu/g (Jofré et al., 2008a). No further recovery of the pathogen was recorded during 3 months of refrigerated storage at 6 °C. These authors (Jofré et al., 2008b) recommended HP treatments at 600 MPa for sliced cooked and dry-cured ham to guarantee the control of *Salmonella* and *L. monocytogenes*.

According to the present results, *Salmonella* was not able to grow in dry-cured ham at 8 °C, and pressurized cells would not recover in the product during refrigerated storage. Considering the expected low level of *Salmonella* contamination in sliced dry-cured ham, HP treatments at 600 MPa for 5 min, which reduced the pathogen counts by more than 4 log units immediately after treatment, would be effective to obtain a safe product, even after storage under temperature abuse conditions.

Table 1
Levels (log cfu/g) of *Salmonella* in pressurized sliced dry-cured ham stored during 60 d at 8 °C.

Time	Non-pressurized	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
8 h	5.48 ± 0.04^{cD}	4.42 ± 0.14^{bcD}	2.94 ± 1.52^{bA}	1.16 ± 1.28^{aA}
1 d	5.53 ± 0.14^{cD}	3.91 ± 0.28^{bD}	1.73 ± 1.18^{aA}	1.36 ± 0.10^{aA}
3 d	5.29 ± 0.05^{dCD}	4.05 ± 0.30^{cD}	2.34 ± 0.19^{bA}	1.10 ± 0.17^{aA}
7 d	4.69 ± 0.35^{dB}	3.23 ± 0.50^{cC}	1.71 ± 0.65^{bA}	PRE^{aA}
15 d	4.86 ± 0.35^{dBC}	3.15 ± 0.20^{cC}	1.84 ± 0.94^{bA}	PRE^{aA}
30 d	4.74 ± 0.11^{dB}	3.15 ± 0.25^{cC}	2.20 ± 0.28^{bA}	PRE^{aA}
45 d	4.12 ± 0.62^{cA}	2.49 ± 0.26^{bB}	1.92 ± 0.66^{bA}	PRE^{aA}
60 d	3.99 ± 0.17^{cA}	1.43 ± 0.51^{bA}	1.33 ± 0.35^{bA}	PRE^{aA}

PRE, presence in 20 g. Limit of detection 10 cfu/g.
Values are the mean ± SD of duplicate determinations in two experiments.
Means within the same row with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same column with different upper-case superscripts differ significantly at $P<0.05$.

3.2. pH and a_w

Values of pH and a_w in pressurized sliced dry-cured ham during 60 d at 8 °C are shown in Table 2. Analysis of variance revealed a significant influence of treatment ($P<0.05$) and time of refrigeration ($P<0.001$) on pH throughout refrigerated storage. A slight increase of pH was observed in pressurized samples after 1 d at 8 °C. Values of pH tended to stabilize in the product and from 45 d no significant differences were recorded in pressurized and control dry-cured ham. Marcos et al. (2005) observed an increase of pH in low-acid fermented sausages after treatment at 300 MPa for 10 min and this effect was attributed to protein denaturation or loss of protons caused by high pressure treatment. In low salt dry-cured hams, pH increases by pressurization at 600 MPa were explained by protein denaturation, resulting in protein unfolding leaving more basic amino acids exposed to the medium (Fulladosa, Serra, Gou, & Arnau, 2009).

High pressure treatment and time of refrigeration had a significant ($P<0.001$) effect on a_w values, which were higher in pressurized dry-cured ham than in non-pressurized samples during refrigerated storage. This difference was maintained for 60 d at 8 °C. An increase in a_w values was reported by Serra et al. (2007a) after pressurization of green hams. Although pressurized dry-cured ham exhibited significantly ($P<0.001$) higher a_w values than non-pressurized samples throughout the refrigeration period, differences were low. Minimal a_w values for the growth of *Salmonella* of approximately 0.95 (Sperber, 1983) have been estimated. Although low a_w values protect microorganisms against inactivation by HP (Patterson, 2005), recovery of sublethally injured bacteria would be inhibited by the lower a_w values in the product.

3.3. Lipid oxidation

The effect of high pressure treatment on TBARS values of sliced vacuum-packed dry-cured ham during refrigerated storage is shown in Table 3. TBARS values were significantly affected by HP treatment ($P<0.05$) and time of refrigeration ($P<0.001$). Differences in lipid oxidation were not significant 1 d after pressurization. However,

Table 2
Values of pH and a_w in pressurized sliced dry-cured ham stored during 60 d at 8 °C.

	Time	Non-pressurized	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
pH	1 d	5.85 ± 0.00^{aB}	5.89 ± 0.01^{bC}	5.91 ± 0.00^{bC}	5.91 ± 0.01^{bC}
	15 d	5.74 ± 0.01^{aA}	5.69 ± 0.04^{aA}	5.71 ± 0.01^{aA}	5.79 ± 0.08^{aAB}
	30 d	5.75 ± 0.01^{bA}	5.70 ± 0.00^{aA}	5.75 ± 0.02^{bA}	5.78 ± 0.01^{bA}
	45 d	5.92 ± 0.04^{aC}	5.93 ± 0.03^{aC}	5.95 ± 0.02^{aC}	5.92 ± 0.02^{aC}
	60 d	5.85 ± 0.01^{aB}	5.84 ± 0.02^{aB}	5.83 ± 0.01^{aB}	5.86 ± 0.02^{aBC}
a_w	1 d	0.891 ± 0.002^{aA}	0.896 ± 0.002^{abB}	0.899 ± 0.002^{bAB}	0.887 ± 0.004^{abA}
	15 d	0.886 ± 0.004^{aA}	0.892 ± 0.002^{bA}	0.897 ± 0.002^{cA}	0.901 ± 0.002^{dB}
	30 d	0.886 ± 0.007^{aA}	0.897 ± 0.003^{bBC}	0.901 ± 0.003^{bBC}	0.901 ± 0.002^{bB}
	45 d	0.887 ± 0.005^{aA}	0.899 ± 0.003^{bBC}	0.901 ± 0.003^{bcBC}	0.903 ± 0.002^{cB}
	60 d	0.891 ± 0.005^{aA}	0.900 ± 0.002^{bC}	0.903 ± 0.001^{bC}	0.901 ± 0.003^{bB}

Values are the mean ± SD of duplicate determinations in two experiments.
Means within the same row with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same column with different upper-case superscripts differ significantly at $P<0.05$.

higher oxidation values were detected in dry-cured ham pressurized samples after 30 and 60 d of refrigeration than in non-pressurized samples. TBARS values tended to increase during refrigerated storage, in agreement with results of Cava et al. (2009) in sliced vacuum-packaged dry-cured ham pressurized at 200–300 MPa for 15–30 min and refrigerated at 4 °C during 90 d. Changes induced by pressurization in lipid oxidation of meat have been described, with increases in TBARS values in high pressurized meat and meat products, indicating that high pressure treatment promoted oxidation (Cheah & Ledward, 1997; Dissing, Bruun–Jensen, & Skibsted, 1997; Orlien, Hansen, & Skibsted, 2000). However, different results have been reported in dry cured products. Higher TBARS values in dry cured ham pressurized at 400 MPa and stored for 39 days were observed by Andrés et al. (2006), showing a decrease in oxidative stability during refrigeration. On the contrary, Campus, Flores, Martínez, and Toldrá (2008) reported opposite results in dry-cured loin, with higher TBARS values in non-pressurized than in pressurized samples after 1 day of storage. These differences were attenuated during refrigerated storage. According to Andrés et al. (2004), high pressure could promote oxidation by breakdown of heme pigments, release of iron and catalysis of oxidation, or the disintegration of membranes making polyunsaturated fatty acid susceptible to oxidation. This pro-oxidant effect of pressurization has been observed in sliced dry-cured Iberian ham (Cava et al., 2009), as well as an increase in TBARS values during storage. However, as reported by these authors, lipid oxidation changes due to pressurization were lower than changes during refrigerated storage in sliced dry-cured meat products.

3.4. Free amino acids (FAA)

A total of 16 FAA were detected and quantified after 1, 30 and 60 d of pressurization. The effect of high pressure treatment on FAA content in sliced dry-cured ham after 60 d at 8 °C is shown in Table 4. High pressure treatments ($P<0.001$) and time of refrigerated storage ($P<0.001$) (data not shown) significantly influenced the content of all the FAA except for Asp and Pro. In sliced dry-cured ham refrigerated at 8 °C during 60 d, total amino acid content in non-pressurized and samples treated at 400 and 600 MPa did not differ significantly, whereas a lower content ($P<0.05$) was detected in samples pressurized at 500 MPa. This tendency was observed for 7 of the 16 FAA analyzed.

High pressure treatment generally produces a reduction in enzymatic activity. Previous results in dry-cured ham showed that proteolysis was not affected by high pressure treatment (Hugas, Garriga, & Monfort, 2002), with no significant differences in the non-protein nitrogen content after treatment at 600 MPa for 10 min. On the contrary, aminopeptidase and dipeptidyl-peptidase activities were reduced by pressurization in dry-cured loin (Campus et al., 2008). This loss of peptidase activity correlated negatively with increasing pressure. As a consequence, the FAA content in untreated samples increased throughout refrigeration at 4 °C for 45 d, whereas this increase was not observed in pressurized dry-cured loin, probably due to the reduction in aminopeptidase activity. The present results indicate only slight changes on FAA content of pressurized and control vacuum-packed dry-cured ham during the 60 d of storage, probably due to the fact that most proteolysis had already occurred during curing.

3.5. Texture

Textural parameters of pressurized sliced dry-cured ham determined with Warner–Braztler and Kramer cells are shown in Table 5. Statistically significant effects of pressure treatment on shear force ($P<0.001$) and maximum force ($P<0.05$) were detected. Pressurization reduced shear resistance determined with the Warner–Bratzler

Table 3
Values of TBARS (mg MDA/kg) in pressurized sliced dry-cured ham stored during 60 d at 8 °C.

Time	Non-pressurized	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
1 d	0.38 ± 0.01^{aA}	0.36 ± 0.01^{aA}	0.40 ± 0.00^{aA}	0.35 ± 0.07^{aA}
30 d	0.46 ± 0.02^{aB}	0.51 ± 0.01^{dC}	0.47 ± 0.01^{bB}	0.49 ± 0.01^{cB}
60 d	0.49 ± 0.01^{bC}	0.45 ± 0.10^{aB}	0.53 ± 0.00^{dC}	0.52 ± 0.01^{cB}

Values are the mean ± SD of duplicate determinations in two experiments.
Means within the same row with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same column with different upper-case superscripts differ significantly at $P<0.05$.

Table 4
Free amino acids content (mg/100 g) in pressurized sliced dry-cured ham after 60 d at 8 °C.

	Non-pressurized	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
Asp	282.0 ± 27.8^{a}	256.1 ± 2.5^{a}	248.7 ± 12.0^{a}	263.3 ± 2.7^{a}
Ser	225.9 ± 0.8^{a}	237.7 ± 2.0^{b}	228.9 ± 0.5^{a}	235.4 ± 2.2^{b}
Glu	284.9 ± 0.8^{b}	280.3 ± 6.2^{b}	265.2 ± 1.7^{a}	279.6 ± 1.6^{b}
Gly	173.7 ± 0.8^{b}	175.4 ± 0.2^{bc}	170.2 ± 0.4^{a}	177.8 ± 0.8^{c}
His	197.9 ± 4.3^{a}	198.9 ± 0.3^{a}	198.1 ± 1.3^{a}	204.4 ± 0.5^{a}
Arg	928.1 ± 1.9^{bc}	939.6 ± 8.0^{c}	901.7 ± 1.8^{a}	915.2 ± 2.3^{ab}
Thr	257.5 ± 1.0^{ab}	259.5 ± 0.7^{b}	253.7 ± 1.4^{a}	260.2 ± 0.7^{b}
Ala	352.4 ± 0.9^{b}	356.3 ± 1.5^{c}	348.5 ± 0.3^{a}	357.5 ± 0.1^{c}
Pro	249.8 ± 1.1^{a}	236.6 ± 2.0^{a}	237.1 ± 8.6^{a}	241.0 ± 10.8^{a}
Tyr	134.4 ± 0.5^{d}	131.5 ± 0.6^{c}	124.8 ± 0.1^{b}	122.9 ± 0.1^{a}
Val	306.9 ± 0.6^{b}	308.7 ± 0.1^{bc}	301.9 ± 0.4^{a}	310.1 ± 0.5^{c}
Met	114.4 ± 1.8^{a}	118.4 ± 0.8^{a}	115.3 ± 0.3^{a}	118.2 ± 0.1^{a}
Lys	557.2 ± 5.2^{ab}	571.8 ± 1.2^{c}	550.9 ± 2.2^{a}	566.7 ± 0.9^{bc}
Ile	242.1 ± 1.1^{b}	244.1 ± 0.1^{b}	238.9 ± 0.3^{a}	244.5 ± 0.5^{b}
Leu	401.8 ± 0.7^{b}	405.8 ± 1.2^{b}	396.2 ± 1.1^{a}	404.4 ± 1.1^{b}
Phe	221.7 ± 0.1^{b}	224.3 ± 0.1^{c}	219.5 ± 0.5^{a}	224.5 ± 0.8^{c}
Total	4930.5 ± 17.3^{b}	4944.9 ± 17.1^{b}	4799.4 ± 16.9^{a}	4925.6 ± 2.2^{b}

Values are the mean ± SD of duplicate determinations in two experiments.
Means within the same row with different lower-case superscripts differ significantly at $P<0.05$.

Table 5
Texture properties of pressurized sliced dry-cured ham stored during 60 d at 8 °C.

		Non-pressurized	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
Shear force (N)	1 d	36.09 ± 2.86^{bBC}	34.49 ± 9.31^{bBC}	23.74 ± 5.75^{aAB}	20.96 ± 9.82^{aA}
	15 d	42.37 ± 14.62^{bCD}	31.89 ± 6.16^{abB}	25.51 ± 4.26^{aB}	20.60 ± 3.83^{aA}
	30 d	54.96 ± 9.57^{cD}	43.16 ± 11.41^{bC}	35.05 ± 7.20^{aC}	32.49 ± 12.85^{aB}
	45 d	27.40 ± 3.28^{bAB}	27.58 ± 2.16^{bB}	24.13 ± 2.36^{abAB}	20.34 ± 2.62^{aA}
	60 d	17.38 ± 2.33^{aA}	16.81 ± 1.32^{aA}	20.77 ± 1.02^{bA}	18.89 ± 1.95^{abA}
Maximum force (N)	1 d	338.90 ± 50.79^{aA}	351.68 ± 97.26^{aA}	341.21 ± 31.65^{aA}	306.74 ± 54.96^{aA}
	15 d	319.13 ± 50.14^{aA}	314.61 ± 62.90^{aA}	397.27 ± 116.68^{aA}	341.48 ± 36.26^{aA}
	30 d	330.47 ± 62.12^{aA}	331.39 ± 37.44^{aA}	385.05 ± 123.71^{aA}	395.19 ± 75.07^{aA}
	45 d	304.56 ± 56.01^{aA}	361.45 ± 54.31^{aA}	357.47 ± 66.09^{aA}	373.22 ± 73.77^{aA}
	60 d	268.34 ± 35.21^{aA}	331.25 ± 57.94^{bA}	318.01 ± 35.13^{abA}	370.65 ± 78.80^{bA}

Values are the mean ± SD of six determinations in two experiments.
Means within the same row with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same column with different upper-case superscripts differ significantly at $P<0.05$.

cell, with values in general significantly ($P<0.05$) lower in dry-cured ham pressurized at 500 or 600 MPa compared to non-pressurized and samples treated at 400 MPa. On the other hand, although a tendency to higher values of maximum force by the Kramer cell was generally found for pressurized dry-cured ham, differences were significant ($P<0.05$) only in pressurized samples at the end of storage at 8 °C. Time of refrigerated storage affected ($P<0.001$) both textural parameters, with lower values at the end of storage in non-pressurized and pressurized dry-cured ham.

Information on the effect of high-pressure treatments on the instrumental texture analysis of dry cured ham is scarce. In cooked ham, pressurization altered meat structure and water distribution, improving the juiciness and increasing tenderness (Bertram, Wu, Straadt, Aagaard, & Aaslyng, 2006). These results are consistent with the present findings, with a decrease in shear force after pressurization, although differences tended to diminish with storage. Crumbliness decreased and fibrousness increased in dry-cured ham pressurized at 600 MPa compared to non-pressurized and ham treated at 400 MPa, but differences did not affect the overall sensory quality of the product (Serra et al., 2007b). Sensory evaluation of restructured dry-cured hams pressurized at 600 MPa (Fulladosa et al., 2009) exhibited a significant increase in hardness, gumminess and fibrousness, also found by instrumental analysis.

3.6. Color

Changes in color parameters (L^*, a^*, and b^*) of vacuum-packed sliced dry-cured ham are shown in Table 6. High pressure treatments significantly ($P<0.001$) influenced L^* (lightness), although differences between pressurized and control samples were low. In general, values of L^* tended to be lower in non-pressurized and pressurized samples at 400 MPa than in dry-cured ham pressurized at 500 and 600 MPa, except 1 day after treatment, with higher ($P<0.05$) values only in samples treated at 400 MPa. Lightness decreased ($P<0.001$) during refrigerated storage in treated and control dry-cured ham, with the changes induced by time of refrigeration higher than those induced by pressurization. Values of a^* (redness) were also significantly ($P<0.001$) affected by pressurization, with lower redness in dry-cured ham treated at 500 and 600 MPa. This tendency was maintained during the 60 d of refrigerated storage. Days of refrigeration significantly ($P<0.01$) influenced redness, with lower values at the end of storage, although differences of this parameter at the end of the 60 days at 8 °C compared to 1 day samples were low. Yellowness values (b^*) were significantly ($P<0.001$) influenced by HP treatments. After 24 h, samples treated at 600 MPa exhibited significantly ($P<0.05$) lower values compared with the other pressurized and non-pressurized samples. Yellowness decreased during storage ($P<0.05$), with lower b^* values in dry cured ham pressurized at 400 MPa and 600 MPa after 60 d at 8 °C.

Modification of the color properties of meat products is commonly induced by HP treatments. Andrés et al. (2004) observed lower L^* values in pressurized dry-cured Iberian ham at 200–400 MPa, whereas treatments at 600–800 MPa led to values similar to controls. Redness also decreased with pressure, although values of a^* increased at 800 MPa with respect to non-pressurized sliced cured ham. Loss of redness of dry-cured ham by HP treatments has been previously reported with the lowest values observed for product exposed to the highest pressure conditions (Andrés et al., 2006; Cava et al., 2009; Morales et al., 2006). According to Andrés et al. (2006), the red pigments of cured meat products are sensitive to high-pressure treatments. Carlez, Veciana-Nogués, and Cheftel (1995) reported that the red color of minced beef turned a much paler pink at pressures ranging between 200 and 350 MPa. This "whitening" effect of pressure was attributed to globin denaturation and/or to heme displacement or release or to the oxidation of the ferrous myoglobin to ferric myoglobin above 400 MPa. Treatments of fuet at 300 MPa for 10 min also reduced significantly ($P<0.05$) b^* values (Marcos et al., 2005). Variations of yellowness in meat products have been related to changes in the chemical state of myoglobin.

The reduction of L^* during storage of pressurized dry-cured loin and the maintenance of differences between treated and control samples were reported (Campus et al., 2008). These differences were attenuated during storage in pressurized and packed in modified atmosphere dry-cured hams (Andrés et al., 2004). In the present work, lightness decreased during storage in pressurized and non-pressurized ham, and a^* and b^* values, although to a lower degree. Lower values of a^* and b^* were maintained after 60 d at 8 °C, mainly

Table 6
Color parameters in pressurized sliced dry-cured ham stored during 60 d at 8 °C.

		Non-pressurized	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
L^*	1 d	47.02 ± 3.86^{aB}	49.34 ± 3.28^{bC}	47.11 ± 2.76^{aA}	46.79 ± 3.24^{aC}
	15 d	44.70 ± 2.63^{aAB}	45.67 ± 3.45^{abB}	47.24 ± 2.96^{bA}	46.54 ± 2.29^{bBC}
	30 d	43.56 ± 3.17^{aA}	43.15 ± 4.16^{aA}	46.94 ± 3.53^{bA}	44.64 ± 2.90^{abABC}
	45 d	44.97 ± 4.02^{abAB}	44.47 ± 2.72^{abAB}	45.78 ± 3.79^{bA}	43.60 ± 3.10^{aA}
	60 d	43.29 ± 3.91^{aA}	43.67 ± 2.73^{aAB}	45.77 ± 2.87^{bA}	44.42 ± 2.95^{abAB}
a^*	1 d	22.28 ± 2.02^{cC}	20.61 ± 2.96^{bB}	19.42 ± 1.64^{abA}	18.50 ± 1.47^{aAB}
	15 d	21.04 ± 1.66^{bABC}	18.95 ± 1.81^{aA}	18.46 ± 1.41^{aA}	18.81 ± 1.25^{aB}
	30 d	20.68 ± 1.92^{bAB}	20.28 ± 2.23^{bAB}	18.33 ± 1.78^{aA}	18.34 ± 1.34^{aAB}
	45 d	19.80 ± 2.29^{aA}	19.77 ± 1.34^{aAB}	18.94 ± 1.75^{aA}	19.35 ± 1.69^{aB}
	60 d	21.36 ± 2.14^{cBC}	20.22 ± 1.84^{bcAB}	19.32 ± 1.39^{bA}	17.50 ± 1.39^{aA}
b^*	1 d	19.23 ± 2.25^{bA}	19.77 ± 2.35^{bB}	18.93 ± 2.50^{bA}	16.93 ± 2.77^{aA}
	15 d	18.92 ± 3.13^{aA}	16.88 ± 3.02^{aA}	17.70 ± 2.56^{aA}	17.23 ± 3.20^{aA}
	30 d	18.37 ± 3.17^{bA}	18.48 ± 2.84^{bAB}	17.81 ± 2.39^{abA}	15.97 ± 3.24^{aA}
	45 d	17.91 ± 3.16^{aA}	18.35 ± 2.45^{aAB}	18.11 ± 2.83^{aA}	17.46 ± 3.96^{aA}
	60 d	19.93 ± 3.85^{bA}	17.42 ± 3.51^{aA}	17.73 ± 3.21^{abA}	15.64 ± 2.65^{aA}

Values are the mean ± SD of twelve determinations in two experiments.
Means within the same row with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same column with different upper-case superscripts differ significantly at $P<0.05$.

in samples treated at higher pressures. Although changes in lightness registered as a result of HP treatments were low in accord with the low changes in lipid oxidation, redness decreased mainly after treatment, whereas lipid oxidation was not affected immediately after pressurization. However, higher TBARS values in dry-cured ham pressurized at 500 and 600 MPa after 60 d of refrigeration at 8 °C were in accord with lower a^* values.

4. Conclusions

High pressure treatments at 400 MPa, 500 MPa and 600 MPa during 5 min were effective in the inactivation of *S. enterica* subsp. *enterica* serovar Enteritidis inoculated in sliced dry-cured vacuum-packaged ham. The pathogen was not detected in samples treated at 600 MPa and maintained at 8 °C after day 7 of refrigeration. The inactivation remained during the 60 d of storage under temperature abuse conditions. According to the results obtained, sublethal injury increased at higher pressures and the damaged cells were not able to recover during storage. Some quality aspects of dry-cured ham were modified by HP treatments. The extent of lipid oxidation was affected by the highest treatments, with higher values of TBARS in pressurized samples. Shear-force was reduced in HP treated samples, but differences were lowered during storage, and maximum force was only slightly changed by pressurization. Lightness was more affected by storage than by HP, whereas redness decreased with the highest treatments. In general, the impact of high pressures at 400–600 MPa during 5 min on the texture and color of sliced vacuum-packaged dry-cured ham was lower than the changes produced during the storage period. *S.* Enteritidis did not recover in the product even under temperature abuse conditions.

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3.2. Inactivation of *Escherichia coli* O157:H7 in dry-cured ham by high-pressure treatments combined with biopreservatives. *Food Control (2013) 31: 508-513.*

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Inactivation of *Escherichia coli* O157:H7 in dry-cured ham by high-pressure treatments combined with biopreservatives

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ABSTRACT

The effect of high-pressure (HP) treatments combined with biopreservatives such as a commercial nisin or pediocin on the survival of *Escherichia coli* O157:H7 and the physicochemical, rheological and color characteristics of sliced dry-cured ham stored under mild temperature abuse conditions (8 °C) was investigated. Immediately after treatments, a synergistic antimicrobial effect was registered when 400 MPa and 500 MPa for 10 min combined with nisin were applied. After 60 d, this synergistic effect was only maintained with the combined treatment of 500 MPa and nisin. Counts of *E. coli* in dry-cured ham were not affected by either nisin or pediocin applied individually, whereas counts in pressurized samples were 3 log units lower than in non-treated dry-cured ham after 60 d of refrigerated storage. Changes in textural parameters caused by pressurization and biopreservatives were minor. Lightness (L^*) values were slightly affected, with lower values in samples pressurized at 500 MPa in combination with bacteriocins and a trend to decrease during storage. Redness (a^*) and yellowness (b^*) were less modified by HP and biopreservatives and tended to diminish during refrigeration. The results obtained in the present work demonstrate the effectiveness of pressurization of sliced dry-cured ham at 500 MPa for 10 min combined with nisin on the inactivation of *E. coli* O157:H7.

1. Introduction

Several types of *Escherichia coli* cause severe gastrointestinal disease in humans. *E. coli* O157:H7 is a serotype of the enterohemorrhagic category of pathogenic *E. coli* strains recognized as Shiga toxin/verocytotoxin producing *E. coli* (VTEC). The pathogen can be shed from the animal in their faecal matter and can contaminate the surfaces of raw meat during the slaughter, dressing and packaging. Human infection may be acquired through the consumption of contaminated food or water. Notified cases of VTEC in humans have been increasing in the EU since 2008, with a total of 4000 confirmed cases in 2010, most of them caused by serogroup O157 (EFSA, 2012). The case fatality rate of VTEC infections was 0.39% in 2010.

Meat and dairy products are the main vehicles of VTEC associated food-borne outbreaks, and are at particular risk because of the high infectivity (Buchanan & Doyle, 1997) and the low infectious dose of VTEC (Kaper, Nataro, & Mobley, 2004). Ready to eat (RTE) meat products can be contaminated by exposure to the environment during slicing and packaging operations (Sheen & Hwang, 2010). *E. coli* can survive at low pH and water activity (a_w) and during refrigeration and freezing. Sliced dry-cured ham, because of the low a_w and high NaCl content, has a long shelf-life at refrigeration temperatures (more than 3 months). The risk of spoilage or pathogen growth increases in case of temperature abuse during storage. *E. coli* outbreaks linked to consumption of fermented sausages have been reported (Ethelberg et al. 2009; Schimmer et al. 2008).

High-pressure (HP) treatment is applied in RTE meat products to eliminate post-processing contamination. Treatments of dry-cured ham at 600 MPa for 6 min prevented the growth of *Enterobacteriaceae* and yeasts and delayed the development of lactic acid bacteria (Garriga, Grèbol, Aymerich, Monfort, & Hugas, 2004). Of particular interest is the use of HP in the elimination of *Listeria monocytogenes* in sliced dry-cured ham due to the ability of the pathogen to grow at refrigeration temperatures and reduced water activity (Hereu, Bover-Cid, Garriga, & Aymerich, 2012; Morales, Calzada, & Nuñez, 2006). Pressurization of Iberian and Serrano ham at 450 MPa for 10 min reduced *L. monocytogenes* populations by 1.50 and 1.16 log cfu/g (Morales et al., 2006) and accelerated pathogen death during refrigerated storage. Higher inactivation rates were achieved in dry-cured ham by HP at 600 MPa during 3 min, with decreases of the pathogen of 2–4 log units depending on the a_w (Hereu et al., 2012). *Salmonella* Enteritidis inactivation by HP in sliced dry-cured ham was investigated by De Alba, Montiel, Bravo, Gaya,

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and Medina (2012), with counts 1.06–4.32 log units lower in samples treated at 400–600 MPa for 5 min compared to the non-pressurized product. According to these authors, *Salmonella* was not able to grow in dry-cured ham at 8 °C, and pressurized cells would not recover in the product during refrigerated storage. HP at 600 MPa reduced *E. coli* O157 numbers in salami by more than 4 log cfu/g but levels increased during storage (Gill & Ramaswamy, 2008).

The effectiveness of mild preservation technologies based on the combination of different processes or antimicrobial factors has been demonstrated (Leistner & Gorris, 1995). The efficacy of bacteriocins can be limited in food systems if applied alone; several bacteriocins have shown additive or synergistic effects when used in combination with other antimicrobial agents or processes such as high hydrostatic pressure. Nisin and pediocin are bacteriocins from lactic acid bacteria with antimicrobial activity against meat associated pathogens (Garriga, Aymerich, Costa, Monfort, & Hugas, 2002; Geornaras et al., 2005; Jofré, Garriga, & Aymerich, 2007). Although bacteriocins have generally no activity against Gram-negative bacteria, combined treatments with HP on the inactivation of *E. coli* have been described to overcome that limitation (Kalchayanand, Sikes, Dunne, & Ray, 1998). Studies on the application of high pressure combined with bacteriocins have been carried out on different meat products (Garriga et al., 2002), and particularly against *L. monocytogenes* in dry-cured ham (Hereu et al., 2012). However, to our knowledge the application of this hurdle technology on sliced dry-cured ham inoculated with *E. coli* O157:H7 has not been investigated.

The aim of this work was to evaluate the combined effect of high-pressure treatments and the biopreservatives nisin and pediocin on the survival of inoculated *E. coli* O157:H7 onto sliced dry-cured ham and to investigate the physicochemical, rheological and color characteristics of this RTE meat product stored under mild temperature abuse conditions (8 °C) during 60 d.

2. Materials and methods

2.1. Microorganisms

E. coli CECT 4972 from the Spanish Type Culture Collection (Valencia, Spain) was kept frozen at −80 °C in tryptic soy yeast extract broth (TSYEB) (Biolife, Milano, Italy) with 30% glycerol. *E. coli* was grown in TSYEB for 18 h at 37 °C.

2.2. Sample preparation

Dry-cured ham was obtained from a local market in Madrid (Spain). Samples (20 g) aseptically sliced were inoculated by spreading 100 μl of *E. coli* culture on the surface to achieve a final population of approximately 10^6 cfu/g. Non-inoculated samples were also prepared to measure pH, a_w, texture and color. Dry-cured ham was sliced into 4-mm-thick slices to determine texture characteristics and 2-mm-thick slices to measure color.

2.3. Addition of biopreservatives

Commercial nisin was prepared in 0.02 M HCl (0.02 g/ml). A commercial product based on pediocin was dissolved in distilled-deionized water. Both biopreservatives were added on the surface of dry-cured ham at 100 IU/g and 0.6% for nisin and pediocin, respectively.

2.4. HP processing

Inoculated and non-inoculated slices were individually vacuum-packaged in double bags of BB325 (Cryovac Grace S.A., Barcelona, Spain). Samples were pressurized at two levels (400 MPa and 500 MPa) and one holding time (10 min) at approx. 12 °C. High-pressure treatments were performed in a high-pressure prototype ACIP 6000 (ACB, Nantes, France) of 3.5-L capacity and 600 MPa maximum working pressure. Water was used as a pressure transmitting medium.

Experimental treatments were nisin, pediocin, 400 MPa for 10 min, 500 MPa for 10 min, 400 MPa for 10 min combined with nisin, 500 MPa for 10 min combined with nisin, 400 MPa for 10 min combined with pediocin, 500 MPa for 10 min combined with pediocin and non-pressurized control group. After pressurization treated and non-treated samples were stored at 8 °C for 60 d. Two independent trials were performed on two different days.

2.5. Microbiological analysis

Samples were analyzed immediately after HP treatments (time 0) and after 8 h, 1, 7, 15, 30, 45 and 60 d. Samples (20 g) were homogenized in 180 ml of 0.1% (wt/vol) sterile peptone water using a homogenizer (IUL, Barcelona, Spain). Decimal dilutions of the homogenate were prepared in the same sterile solution. *E. coli* O157:H7 population was determined on duplicate plates of violet red bile agar (VRBA) (Oxoid Ltd., Basingstoke, England) incubated at 37 °C for 24 h.

2.6. pH and a_w

pH determinations were taken using a pH-meter (model GPL22, Crison Instruments, Barcelona, Spain). Samples (10 g) of dry-cured ham were homogenized in 90 ml of distilled water in stomacher bags for 90 s. Water activity (a_w) was measured in *Quadriceps femoris* and *Biceps femoris* muscles using the AquaLab Series 3 equipment (Decagon Devices, Inc., Pullman, WA, USA). Samples were taken 1, 30 and 60 d after treatments. Two measurements were performed per sample.

2.7. Textural determinations

Sliced dry-cured ham texture was determined using an Instron Compression Tester 4301 (Instron Ltd., Barcelona, Spain) controlled by the BlueHill V2.0 software, with a load cell of 1000 N and crosshead speed of 100 mm/min. The shear strength (*N*) required to shear the sample was examined using a Warner-Bratzler blade and hardness (*N*), considered as the maximum force required to compress the sample, was determined with a Kramer cell. Three measurements were taken at each muscle (*Quadriceps femoris* and *Biceps femoris*) per sample in dry-cured ham 1, 30 and 60 d after treatments.

2.8. Color measurements

Surface color of sliced dry-cured ham was measured six times on each muscle (*Q. femoris* and *B. femoris*) per sample using a Minolta CM 700d Chromometer (Minolta Camera Co., Osaka, Japan). L^* (lightness, intensity of white color), a^* (+*a*, red; −*a*, green) and b^* (+*b*, yellow; −*b*, blue) were determined 1, 30 and 60 d after treatments.

2.9. Statistical analysis

Data were subjected to analysis of variance (ANOVA) by means of SPSS program Win 12.0 software (SPSS Inc., Chicago, IL, USA), with treatment and time of storage as main effects. Significant differences between means were assessed by Tukey test with a confidence interval of 95%.

3. Results and discussion

3.1. Inactivation of E. coli O157:H7

The behavior of *E. coli* O157:H7 in sliced vacuum-packaged dry-cured ham subjected to combined treatments of high pressure with biopreservatives and stored under refrigeration at 8 °C during 60 d is shown in Fig. 1. *E. coli* mean initial counts in inoculated dry-cured ham were 6.13 log cfu/g. Immediately after pressurization, reductions of *E. coli* in VRBA were 0.25 and 1.28 log cfu/g in pressurized ham at 400 MPa and 500 MPa. Counts of *E. coli* were not affected by nisin or pediocin applied individually. The reductions in *E. coli* counts in pressurized samples at 400 MPa and 500 MPa combined with nisin were 1.32 and 1.81 log cfu/g, respectively. The inactivation achieved by the combination of HP and nisin was slightly higher than the sum of the inactivation obtained with each single treatment, so a synergistic effect was observed. On the contrary, pressurization combined with pediocin did not result in increased antimicrobial activity against the pathogen.

During the storage under refrigeration at 8 °C, counts of *E. coli* O157:H7 only diminished by 0.43 log cfu/g in non-treated samples, indicating the ability of *E. coli* to survive in dry-cured ham during prolonged storage. *E. coli* O157:H7 levels decreased in sliced dry-cured ham by 0.6–1.6 log units during storage for 28 d at 2 °C (Ng, Langlois, & Moody, 1997). Its ability to survive in other meat products has been reported, with reductions in fermented sausages between 0.15 and 0.95 log units after storage at 4 °C (Holck et al., 2011).

Higher decreases were observed throughout refrigeration in treated dry-cured ham. At the end of the storage period at 8 °C, VRBA counts were 3.28 and 3.18 log cfu/g lower in pressurized samples at 400 MPa and 500 MPa, respectively, than in non-pressurized samples. In treated samples at 400 MPa and 500 MPa combined with nisin, differences were 3.07 and 4.00 log cfu/g. In treated samples at 400 MPa and 500 MPa combined with pediocin, theses differences in *E. coli* population were 2.61 and 3.06 log cfu/g, respectively. The synergistic effect of pressurization at 500 MPa combined with nisin, that achieved a reduction of 0.82 log units higher that the observed with HP treatments after 60 d of storage at 8 °C, was observed throughout storage. No antimicrobial activity due to bacteriocins was detected in dry-cured ham when the biopreservatives were applied individually, with levels in 60 d samples that did not differ significantly from those of control dry-cured ham.

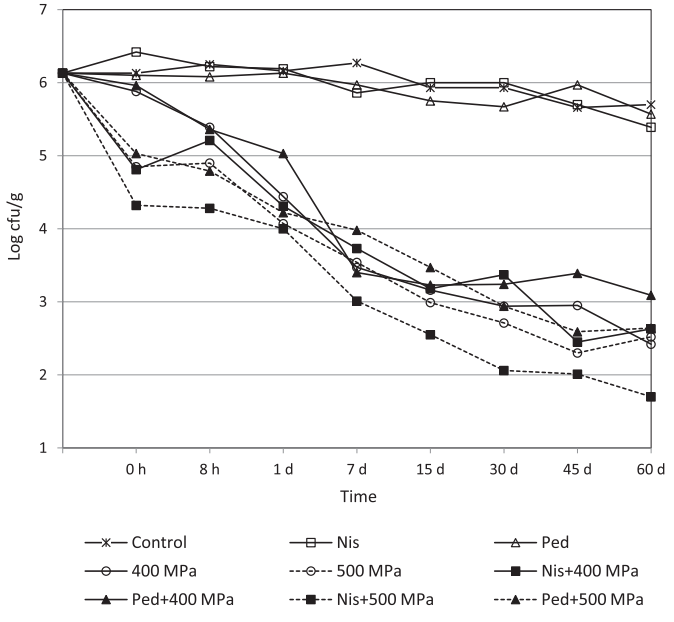

Fig. 1. Behavior of *Escherichia coli* O157:H7 in sliced dry-cured ham stored during 60 d at 8 °C.

Sublethally damaged *E. coli* were not able to grow during the 60 d of refrigeration, decreasing both in non-pressurized and pressurized dry-cured ham, although this decline in counts was accelerated in samples treated with high pressures. The conditions of low water activity and high NaCl content found in dry-cured ham make an adverse environment that did not favor the recovery of stressed cells. This fact was also observed with other pathogens as *S.* Enteritidis (De Alba et al., 2012) and *L. monocytogenes* (Bover-Cid, Belletti, Garriga, & Aymerich, 2011).

Viability loss of *E. coli* O157:H7 by pressurization was low, even in dry-cured ham, if compared to *Salmonella* or *L. monocytogenes*. Although Gram-negative bacteria are in general more sensitive to pressurization than Gram-positives, *E. coli* O157:H7 is considered a pressure-resistant serotype (Benito, Ventoura, Casadei, Robinson, & Mackey, 1999). When assayed in a meat model system (Garriga et al., 2002), *E. coli* was more resistant to HP than other Gram-positive and Gram-negative species. The reductions in *E. coli* O157:H7 population in dry-cured ham at 400 or 500 MPa were lower than the attained with another strain of *E. coli* O157:H7 by treatments at 300 or 500 MPa in cheese (Rodríguez, Arqués, Nuñez, Gaya, & Medina, 2005). Moreover, the higher sensitivity of non-O157:H7 *E. coli* strains to pressurization have been reported in cheese (Capellas, Mor-Mur, Sendra, Pla, & Guamis, 1996; O'Reilly, O'Connor, Kelly, Beresford, & Murphy, 2000). Differences in *E. coli* inactivation by high pressures in different meat products could be attributed partially to differences in a_w, with higher inactivation rates at higher values of this characteristic (Palou, Lopez-Malo, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1997). Products with low water activity protect bacteria from the lethality caused by HP treatments (Oxen & Knorr, 1993).

Gram-negative bacteria are normally insensitive to some antimicrobial agents as lysozyme or nisin and other lactic acid bacteria bacteriocins (Hauben, Wuytack, Soontjens, & Michiels, 1996). Pressurization treatments induce sublethally injured *E. coli* O157:H7 cells that are more sensitive to nisin or pediocin when the bacteriocin was added before HP treatment (Hauben et al., 1996; Kalchayanand et al., 1998). A different behavior of *E. coli* O157:H7 after pressurization in the presence of nisin or pediocin was observed in the present work, with enhanced antimicrobial activity in dry-cured ham in the presence of nisin, but not of pediocin. In agreement with our results, pressurization at 400 MPa for 10 min at 17 °C of a model meat system with nisin A led to a greater inactivation of *E. coli* population (>6 log units) that persisted throughout 60 days of storage at 4 °C, suggesting that the injured survivors became sensitive to nisin after pressurization (Garriga et al., 2002). The antimicrobial activity observed was higher than the inactivation of the pathogen if sakacin K, enterocins A and B, or pediocin AcH were added. Other bacteriocins have been assayed in combined treatments against *E. coli*. The extract of *Lactobacillus casei* OSY-LB6A acted synergistically in sausage when applied in combination with HP against pressure-resistant *E. coli* O157 strains (Chung & Yousef, 2010), the presence of the antimicrobial extract damaged cellular components during pressurization.

Combined hurdles of biopreservation and HP treatments have been proposed as anti-*Listeria* strategy during storage of RTE cured meat products (Hereu et al., 2012). According to these authors, nisin applied onto the surface of dry-cured ham slices reduced *L. monocytogenes* counts approx. 1 log unit after treatment and 3 log units more during 60 d of refrigeration at 8 °C. The inactivation caused by HP in combination with nisin was increased, although a_w

values in the product of 0.88 exerted a baroprotective effect on the pathogen. Other studies in meat products demonstrated the enhancement of *L. monocytogenes* inactivation by *in situ*-produced pediocin in fermented sausages (Foegeding, Thomas, Pilkington, & Klaenhammer, 1992). *Listeria* proliferation in different types of fermented sausages has been also controlled by pediocins produced by *Pediococcus acidilactici* and enterocins produced by *Enterococcus faecium* CTC492 that provided an additional hurdle against the growth of the pathogen (Aymerich, Garriga, Jofré, Martín, & Monfort, 2006). The effectiveness of the combination of 400 MPa and nisin against *Salmonella* and *L. monocytogenes* was also demonstrated in sliced cooked ham by Aymerich, Jofré, Garriga, and Hugas (2005), although the growth of these pathogens was not completely inhibited. In dry-cured ham, pressurization at 600 MPa combined with bacteriocins kept *Salmonella* absent in 25 g samples during 120 d (Jofré, Aymerich, Monfort, & Garriga, 2008).

Our results indicate that treatments at 400 MPa and 500 MPa during 10 min resulted effective on the inactivation of *E. coli* O157:H7 inoculated in sliced dry-cured ham, and that a slight synergistic antimicrobial effect was attained when combined treatments of high pressure at 500 MPa with nisin were applied, improving the efficacy of HP treatments in the product.

3.2. pH and a_w

Values of pH and a_w in dry-cured ham are shown in Table 1. An increase ($P < 0.05$) in pH values was observed in sliced dry-cured ham pressurized at 400 MPa and 500 MPa one day after treatments. This effect could be attributed to protein denaturation or loss of protons caused by pressurization (Marcos, Aymerich, & Garriga, 2005). The values of pH tended to decrease ($P < 0.001$) during refrigeration at 8 °C, although differences were low at the end of the storage period. These results are in accordance with De Alba et al. (2012) who observed that differences in pH of pressurized dry-cured ham at 400–600 MPa tended to attenuate during refrigerated storage.

Higher values ($P < 0.05$) of a_w were recorded in treated samples compared with control, one day after treatments. Values of a_w diminished ($P < 0.05$) in samples treated at 400 MPa and 500 MPa at the end of refrigerated period and increased in the rest of samples, although the maximum difference between non-treated and treated sliced dry-cured ham was only 0.03 units. Low a_w values and/or high solute concentration have been recognized to exert a baroprotective effect and reduce the extent of bacterial inactivation induced by HP (Patterson, 2005). In the sliced dry-cured ham used in this work, with values of a_w between 0.882 and 0.907, recovery of sublethally injured cells would be impeded by the lower a_w in the product.

Table 1
Values of pH and a_w of sliced dry-cured ham stored during 60 d at 8 °C.

	Treatment	1 d	30 d	60 d
pH	Control	5.87 ± 0.05^{bcC}	5.68 ± 0.04^{abA}	5.82 ± 0.06^{bcdeB}
	Nisin	5.84 ± 0.02^{abB}	5.76 ± 0.04^{cA}	5.78 ± 0.09^{abA}
	Pediocin	5.94 ± 0.16^{eB}	5.83 ± 0.14^{dA}	5.85 ± 0.04^{efA}
	400 MPa	5.93 ± 0.06^{deC}	5.76 ± 0.03^{cA}	5.83 ± 0.11^{cdeB}
	500 MPa	5.90 ± 0.02^{cdC}	5.78 ± 0.13^{cdA}	5.84 ± 0.02^{deB}
	Nis + 400 MPa	5.84 ± 0.02^{abB}	5.75 ± 0.07^{cA}	5.78 ± 0.05^{abcA}
	Ped + 400 MPa	5.81 ± 0.06^{aA}	5.76 ± 0.11^{cA}	5.79 ± 0.05^{bcdA}
	Nis + 500 MPa	5.87 ± 0.05^{bcC}	5.64 ± 0.02^{aA}	5.73 ± 0.10^{aB}
	Ped + 500 MPa	5.86 ± 0.06^{bcB}	5.72 ± 0.11^{bcA}	5.90 ± 0.15^{fB}
a_w	Control	0.884 ± 0.009^{aAB}	0.887 ± 0.004^{aB}	0.882 ± 0.011^{aA}
	Nisin	0.899 ± 0.006^{cA}	0.897 ± 0.005^{bcA}	0.906 ± 0.008^{efB}
	Pediocin	0.893 ± 0.005^{bA}	0.897 ± 0.006^{bcB}	0.904 ± 0.007^{deC}
	400 MPa	0.903 ± 0.004^{deC}	0.895 ± 0.005^{bA}	0.897 ± 0.006^{bB}
	500 MPa	0.904 ± 0.007^{eC}	0.897 ± 0.004^{cA}	0.901 ± 0.005^{cB}
	Nis + 400 MPa	0.904 ± 0.007^{eB}	0.901 ± 0.012^{deA}	0.907 ± 0.006^{fC}
	Ped + 400 MPa	0.901 ± 0.011^{cdAB}	0.900 ± 0.006^{dA}	0.902 ± 0.009^{cdB}
	Nis + 500 MPa	0.900 ± 0.012^{cdA}	0.902 ± 0.008^{eB}	0.907 ± 0.008^{fC}
	Ped + 500 MPa	0.901 ± 0.013^{cdA}	0.902 ± 0.009^{eA}	0.901 ± 0.002^{cA}

Values are the mean ± SD.
[a,b,c,d,e,f] Means within the same column with different superscript differ significantly at $P < 0.05$.
[A,B,C] Means within the same row with different superscript differ significantly at $P < 0.05$.

3.3. Texture

Textural properties of sliced dry-cured ham stored during 60 d at 8 °C are shown in Table 2. Shear strength values (*N*) determined with Warner-Bratzler blade were significantly ($P < 0.05$) lower only in dry-cured ham with bacteriocins applied individually or at 500 MPa regarding non-treated samples one day after treatments. During refrigeration at 8 °C, values of shear strength tended to increase, although variations in values did not show a clear trend. Similar results were observed for hardness of sliced dry-cured ham determined as maximum force (*N*) with Kramer cell, with higher ($P < 0.05$) values in samples treated with nisin or with the combination of 400 MPa and nisin or pediocin than in control dry-cured ham, and without a clear tendency during refrigerated storage at 8 °C.

High pressure induces alteration in meat structure and water distribution affecting juiciness and increasing tenderness, depending on the product composition, pressure level and pressure/temperature combination. Serra et al. (2007) observed that 600 MPa increased fibrousness but not modified hardness of dry-cured hams, although the overall sensory quality was not negatively affected. Increases in hardness and chewiness of dry-cured ham pressurized at 600 MPa determined by sensory analysis were reported (Clariana et al., 2011; Fuentes, Ventanas, Morcuende, Estévez, & Ventanas, 2010). Hardness, gumminess and fibrousness detected instrumentally also increased in the pressurized product (Fulladosa, Serra, Gou, & Arnau, 2009). These changes could be originated by partial denaturation of muscle proteins and their modification as a result of protein oxidation (Fuentes et al., 2010). Dry-cured ham treated at 500 or 600 MPa exhibited lower shear resistance than samples pressurized at 400 MPa, whereas the maximum force to compress the sample was only slightly modified by pressurization (De Alba et al., 2012). According to these authors, time of refrigerated storage affected dry-cured ham texture, with lower values for shear resistance and hardness at the end of the refrigeration storage in non-pressurized and pressurized dry-cured ham.

3.4. Color

The effect of treatments on color (CIE L^*, a^*, b^*) of vacuum-packaged sliced dry-cured ham is shown in Table 3. Changes in L^* were low, with values one day after treatment significantly ($P < 0.05$) lower than in control dry-cured ham in samples treated with pediocin and samples pressurized at 500 MPa in the presence of bacteriocins. Lightness tended to decrease in treated and control dry-cured ham samples during the refrigerated storage at 8 °C. Redness (a^*) values of dry-cured ham were slightly affected after treatments, and tended to decrease with refrigeration. Yellowness (b^*) values did not change significantly in dry-cured ham pressurized or treated with bacteriocins, with the exception of an increase ($P < 0.05$) in b^* values of dry-cured ham pressurized at 400 MPa with pediocin. Lower values for yellowness were recorded in most cases after 60 d of refrigerated storage.

High-pressure treatments induce changes in the color of meat and meat products. Increases in lightness (L^*) by treatments above

Table 2
Texture properties of sliced dry-cured ham stored during 60 d at 8 °C.

	Treatment	1 d	30 d	60 d
Shear force (*N*)	Control	35.62 ± 11.95^{cA}	32.48 ± 5.18^{bcA}	38.37 ± 9.42^{dA}
	Nisin	27.05 ± 11.11^{abA}	34.12 ± 3.57^{abB}	38.16 ± 10.10^{dB}
	Pediocin	25.17 ± 7.96^{aA}	41.90 ± 3.33^{eB}	30.74 ± 12.76^{bcA}
	400 MPa	37.13 ± 11.77^{cA}	34.11 ± 5.40^{abA}	34.32 ± 9.96^{cdA}
	500 MPa	27.31 ± 8.26^{abA}	36.35 ± 14.65^{bcdB}	30.17 ± 6.94^{abcA}
	Nis + 400 MPa	31.81 ± 7.68^{abcB}	39.04 ± 7.33^{cdeC}	26.30 ± 5.51^{abA}
	Ped + 400 MPa	30.92 ± 8.85^{abcA}	40.12 ± 4.33^{deB}	29.10 ± 8.42^{abcA}
	Nis + 500 MPa	32.45 ± 13.64^{bcB}	36.19 ± 12.47^{bcdB}	23.05 ± 5.69^{aA}
	Ped + 500 MPa	33.35 ± 6.42^{bcB}	30.91 ± 8.05^{aAB}	28.87 ± 8.02^{abcA}
Hardness (*N*)	Control	336.78 ± 133.84^{aA}	338.67 ± 35.40^{abA}	393.76 ± 46.29^{aA}
	Nisin	421.51 ± 116.39^{bcdB}	386.44 ± 62.46^{bcA}	362.64 ± 51.10^{aA}
	Pediocin	345.66 ± 97.11^{aA}	311.71 ± 59.06^{aA}	396.45 ± 45.56^{aA}
	400 MPa	353.62 ± 14.96^{aAB}	355.99 ± 50.13^{abcA}	400.06 ± 71.23^{aB}
	500 MPa	391.44 ± 99.88^{abcA}	533.40 ± 54.01^{eB}	387.16 ± 57.85^{aA}
	Nis + 400 MPa	450.35 ± 78.66^{cdA}	408.76 ± 70.34^{cdA}	511.38 ± 106.67^{bA}
	Ped + 400 MPa	467.27 ± 63.79^{dB}	453.15 ± 81.99^{dAB}	425.47 ± 49.23^{aA}
	Nis + 500 MPa	398.32 ± 77.25^{abcA}	552.48 ± 98.97^{eA}	413.52 ± 96.10^{aA}
	Ped + 500 MPa	377.08 ± 120.92^{abA}	389.29 ± 50.20^{bcA}	413.45 ± 95.09^{aA}

Values are the mean ± SD.
[a,b,c,d,] Means within the same column with different superscript differ significantly at $P < 0.05$.
[A,B,C] Means within the same row with different superscript differ significantly at $P < 0.05$.

200 MPa is the most often reported effect on raw meat color (Simonin, Duranton, & de Lamballerie, 2012). The color of raw cured meats is less affected by this technology. In sliced dry-cured ham, Andrés, Møller, Adamsen, and Skibsted (2004) reported significant L^* decreases at 200–400 MPa but not at 600–800 MPa. L^* and a^* were not affected by 450 MPa for 10 min in Iberian and Serrano cured hams (Morales et al., 2006), and 400 MPa treatments did not change redness values after treatment and refrigerated storage (Cava, Ladero, Gonzalez, Carrasco, & Ramirez, 2009; Clariana, Guerrero, Sárraga, & García-Regueiro, 2012). However, loss of redness of dry-cured ham by HP treatments also has been reported and the lowest values for a^* were observed for the product exposed to the highest pressure conditions (Andrés, Adamsen, Møller, Ruiz, & Skibsted, 2006; De Alba et al., 2012). The effect of HP on color was influenced by treatment intensity, as redness of dry-cured hams decreases with increasing pressures, especially above 400 MPa. According to Andrés et al. (2006), the red pigments of cured meat products are sensitive to high-pressure treatments. Decreases in redness by pressurization have been related to some changes in the myoglobin molecule, such as oxidation of the ferrous myoglobin to ferric myoglobin and an increase in the proportion of metmyoglobin at the expense of oxymyoglobin (Carlez, Veciana-Nogués, & Cheftel, 1995). Saccani, Parolari, Tanzi, and Rabbuti (2004) reported a slight discoloration of dry-cured ham after treatment at 600 MPa for 9 min, and similar results were observed by Andrés et al. (2004) in ham subjected to different pressures. According to Carlez et al. (1995), meat discoloration by pressurization may result from a whitening effect due to globin denaturation and/or to heme displacement or release, and to the oxidation of ferrous myoglobin to ferric metmyoglobin, which is undesirable and occurs progressively in fresh meat along storage under refrigeration. Curing would confer higher color stability and protect dry-cured ham against pigment oxidation by the high-pressure treatments (Serra et al., 2007), being nitrosylmyoglobin pigment responsible for dry-cured meat color (Moller & Skibsted, 2002).

Table 3
Color parameters of sliced dry-cured ham stored during 60 d at 8 °C.

	Treatment	1 d	30 d	60 d
L^*	Control	44.73 ± 4.41^{cB}	40.53 ± 4.61^{abcA}	38.58 ± 5.17^{abA}
	Nisin	42.13 ± 3.21^{abcB}	42.45 ± 4.70^{bcdB}	38.29 ± 4.15^{aA}
	Pediocin	41.75 ± 3.66^{abB}	39.26 ± 3.25^{aA}	39.57 ± 4.42^{abA}
	400 MPa	42.66 ± 3.30^{abcB}	39.81 ± 5.33^{abA}	39.93 ± 4.47^{abcA}
	500 MPa	44.03 ± 4.55^{bcA}	44.22 ± 4.10^{dA}	43.11 ± 5.20^{cdA}
	Nis + 400 MPa	44.19 ± 5.24^{bcB}	43.12 ± 3.85^{cdAB}	41.31 ± 3.92^{abcdA}
	Ped + 400 MPa	44.59 ± 5.04^{cB}	42.16 ± 3.41^{bcdA}	44.35 ± 6.12^{dAB}
	Nis + 500 MPa	40.48 ± 4.10^{aA}	44.54 ± 5.13^{dB}	42.81 ± 4.20^{cdB}
	Ped + 500 MPa	40.83 ± 5.43^{aA}	42.39 ± 3.26^{bcdA}	41.49 ± 5.20^{bcdA}
a^*	Control	19.41 ± 2.48^{abA}	21.02 ± 2.55^{cB}	19.67 ± 2.55^{eA}
	Nisin	21.41 ± 1.86^{dC}	20.18 ± 2.07^{cB}	18.83 ± 3.00^{cdeA}
	Pediocin	19.92 ± 2.18^{bcdAB}	20.45 ± 2.84^{cB}	18.59 ± 2.92^{cdeA}
	400 MPa	18.16 ± 2.19^{aA}	20.08 ± 2.10^{bcB}	16.99 ± 3.83^{bcA}
	500 MPa	19.37 ± 1.77^{abC}	18.04 ± 2.39^{aB}	16.30 ± 3.53^{abA}
	Nis + 400 MPa	19.77 ± 2.38^{abcA}	19.85 ± 2.23^{bcA}	19.04 ± 2.24^{deA}
	Ped + 400 MPa	21.21 ± 2.26^{cdC}	19.71 ± 1.52^{abcB}	17.46 ± 2.83^{bcdA}
	Nis + 500 MPa	19.62 ± 2.11^{abcB}	20.00 ± 2.31^{bcB}	18.04 ± 2.26^{bcdeA}
	Ped + 500 MPa	20.82 ± 2.29^{bcdC}	18.42 ± 2.34^{abB}	14.98 ± 1.92^{aA}
b^*	Control	19.75 ± 3.32^{bcB}	20.05 ± 4.56^{abB}	17.85 ± 3.34^{bA}
	Nisin	20.18 ± 3.14^{cB}	19.52 ± 3.38^{abAB}	17.55 ± 3.28^{bA}
	Pediocin	18.71 ± 2.52^{abcA}	18.31 ± 4.42^{aA}	17.49 ± 2.66^{bA}
	400 MPa	17.87 ± 3.55^{abAB}	19.31 ± 3.17^{abB}	16.75 ± 2.78^{abA}
	500 MPa	19.00 ± 4.33^{abcA}	19.33 ± 3.61^{abA}	19.13 ± 4.78^{bA}
	Nis + 400 MPa	20.04 ± 2.94^{bcAB}	20.40 ± 2.83^{abB}	18.53 ± 2.92^{bA}
	Ped + 400 MPa	22.40 ± 4.00^{dC}	19.75 ± 2.56^{abB}	17.85 ± 3.41^{bA}
	Nis + 500 MPa	16.92 ± 3.52^{aA}	21.29 ± 4.50^{bB}	18.68 ± 5.15^{bA}
	Ped + 500 MPa	19.81 ± 3.29^{bcB}	19.37 ± 3.40^{abB}	14.84 ± 4.21^{aA}

Values are the mean ± SD.
[a,b,c,d,e] Means within the same column with different superscript differ significantly at $P < 0.05$.
[A,B,C] Means within the same row with different superscript differ significantly at $P < 0.05$.

In the present work, changes in color properties of dry-cured ham subjected to pressurization at 400 or 500 MPa individually or in combination with biopreservatives were low, and values of L^*, a^* and b^* tended to decrease during refrigerated storage.

4. Conclusions

High-pressure treatments at 400 MPa and 500 MPa during 10 min resulted effective on the inactivation of *E. coli* O157:H7 inoculated in sliced dry-cured ham, although the reductions achieved were low. Treatments with biopreservatives nisin or pediocin applied individually did not affect the survival of *E. coli* O157:H7. However, when combined treatments of high pressure with nisin were applied, a slight antimicrobial synergistic effect of approximately one log unit was observed. The synergy between 500 MPa and nisin was maintained after 60 d at 8 °C, providing a wider margin of safety in the control of *E. coli* O157:H7 during the storage

of sliced dry-cured ham and improving the efficacy of HP treatments in the product at lower pressures. Pressurization combined with biopreservatives modified slightly other aspects of quality of dry-cured ham, such as texture and color.

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3.3. Inactivation of *Listeria monocytogenes* and *Salmonella* Enteritidis in dry-cured ham by combined treatments of high pressure and the lactoperoxidase system or lactoferrin. *Innovative Food Science & Emerging Technologies* (Manuscrito en preparación).

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Inactivation of *Listeria monocytogenes* and *Salmonella* Enteritidis in dry-cured ham by combined treatments of high pressure and the lactoperoxidase system or lactoferrin

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Running title: High pressure and lactoferrin or lactoperoxidase against *Listeria monocytogenes* and *Salmonella* Enteritidis in dry-cured ham

Abstract

The effect of high hydrostatic pressure (HHP) treatment at 450 MPa for 10 min combined with the lactoperoxidase system (LP system) or lactoferrin (LF) on the survival of *Listeria monocytogenes* and *Salmonella* Enteritidis and the characteristics of sliced dry-cured-ham stored during 60 d at 8 ºC were investigated. Levels of *L. monocytogenes* decreased by 0.58 log cfu/g after 450 MPa whereas the pathogen was not affected by the LP system or LF applied individually. A synergistic antimicrobial activity was detected on *S.* Enteritidis treated at 450 MPa in combination with the LP system or LF. Lightness (*L**) of dry-cured-ham increased with combined treatments, although differences were attenuated at the end of storage. Redness (*a**) values decreased by HHP applied alone or in combination with antimicrobials and an increase of yellowness (*b**) was observed with LF after 60 d at 8 ºC. Differences in textural properties were attenuated at the end of storage.

Industrial relevance: Antimicrobial activity of high pressure treatment in combination with the lactoperoxidase system or lactoferrin against *L. monocytogenes* and *S.* Enteritidis in sliced dry-cured ham stored during 60 d at 8 ºC is presented. Color and texture were slightly modified by treatments and the safety of the product was improved.

Keywords: *L. monocytogenes, S.* Enteritidis, dry-cured ham, high pressure, lactoperoxidase system, lactoferrin.

1. Introduction

Sliced dry-cured ham is a ready to eat (RTE) meat product that can be contaminated during slicing and packaging operations (Sheen & Hwang, 2010). Although considered a product with a long shelf-life at refrigeration temperature, the risk of spoilage or food-pathogen growth increases in the case of temperature abuse conditions over a long storage period (Cava, Ladero, González, Carrasco, & Ramírez, 2009). *Listeria monocytogenes* can multiply at refrigeration temperatures and reduced water activity (Hereu, Bover-Cid, Garriga, & Aymerich, 2012), fact which makes the occurrence in RTE foods with a relatively long shelf life of particular concern. *L. monocytogenes* has been reported to be capable of rapid growth on a variety of meat products at 7 ºC (Beumer, Te Giffel, De Boer, & Rombouts, 1996). Based on the known characteristics of this microorganism and the disease caused, listeriosis, the FDA (Food and Drug Administration, EE.UU.) maintains a policy of "zero tolerance" for *L. monocytogenes* in RTE foods. The European Union regulations have established a limit of 100 cfu/g for RTE foods placed in the market during their shelf-life (CE, 2005). The foods most frequently associated with *Listeria* outbreaks have been identified as RTE foods, smoked fish and other fishery products, followed by meat products and cheese (Lianou & Sofos, 2007). *Salmonella* has long been recognized as an important zoonotic pathogen of economic significance in animals and humans. Transmission often occurs when organisms are introduced in food preparation areas and are allowed to multiply in food due to inadequate storage temperatures, inadequate cooking or cross contamination of RTE foods. US and EC food regulations require *Salmonella* to be absent from ready-to-eat food products that are not intended to be heated before being consumed (Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. OJ L 322, 7.12.2007, pp. 12–29).

The lactoperoxidase (LP) system is part of the immune system innate defence mechanism against foreign microorganisms and can be found in mammalian

secretions such as milk, tears and saliva. This system consists in three components, lactoperoxidase, thiocyanate (SCN^-) and hydrogen peroxide. The LP system activation occurs only in the presence of thiocyanate and hydrogen peroxide. Catalysis by lactoperoxidase generates active intermediates with antimicrobial properties. The presence of glucose oxidase (GOX) and its substrate (glucose) allows hydrogen peroxide required by the LP system to be continuously generated and replenished (Seifu, Buys, & Donkin, 2005). It has been reported that a more sustained inhibitory effect is obtained if the hydrogen peroxide is generated *in situ* by added glucosa oxidase (GOX) and glucose (Earnshaw, Banks, Defrise, & Francotte, 1989). The antibacterial action of the LP system is thought to be due to oxidation of essential sulfhydryl groups in proteins by intermediary oxidation products of thiocyanate resulting in altered cellular function, e.g., membrane integrity, transport systems, inhibition of metabolic enzymes (Reiter & Harnulv, 1984; Thomas, 1985; Wolfson & Sumner, 1993). Different groups of bacteria show a varying degree of sensitivity to the LP system. This difference of sensitivity can probably be explained by the differences in cell wall structure and their different barrier properties (De Wit & vanHooydonk, 1996; Reiter & Harnulv, 1984). The LP system show variable effects related to bacterial species that are present in the food being treated, the storage temperature of the food, the initial inoculum and the culture medium (Seifu et al., 2005). Some studies about the LP system applied individually (Kennedy, O´Rourke, McLay, & Simmonds, 2000; Elliot, McLay, Kennedy, & Simmonds, 2004) or combined with monolaurin (McLay, Kennedy, O´Rourke, Elliot, & Simmonds, 2002) have been carried out to assess the effect of these antimicrobial compounds on different foodborne bacteria in red meat.

Bovine lactoferrin (LF) is an iron-binding glycoprotein of approximately 80 kDa molecular mass, closely related to transferrins. It is a key element of the innate host defence system in mammals, and exerts antimicrobial activities against a broad range of Gram-positive and Gram-negative bacteria (Jenssen & Hancock, 2009). LF iron-sequestering ability is at the basis of its bacteriostatic effect, whereas the

bactericidal activity is mediated by binding to or altering bacteria cell wall components, such as lipopolysaccharide (LPS) molecules in Gram-negative bacteria and teichoic acid (TA) or lipoteichoic (LTA) acids in Gram-positive bacteria, taking into account depolarization, loss of membrane integrity, and loss of pH gradient (Ellison, 1994; Vorland, Ulvatne, Rekdal, & Svendsen, 1999). Studies both buffer and broth have been carried out to test antimicrobial activity of LF and its derivatives on spoilage and foodborne bacteria (Del Olmo, Morales, & Nuñez, 2008; Del Olmo, Calzada, & Nuñez, 2010). The effect of these antimicrobial compounds has been also investigated in meat products (Del Olmo, Morales, & Nuñez, 2009).

High hydrostatic pressure (HHP) is applied to eliminate post-processing contamination of RTE meat products. This technology, applied individually (Jofré, Aymerich, Grébol, & Garriga, 2009; De Alba, Montiel, Bravo, Gaya, & Medina, 2012a; De Alba, Bravo, & Medina, 2012b) or in combination with different antimicrobials such as the LP system (García-Graells, Van Opstal, Vanmuysen, & Michiels, 2003; Montiel, Bravo, De Alba, Gaya, & Medina, 2012), LF or its derivates (Del Olmo, Calzada, & Nuñez, 2012a, b), bacteriocins such as nisin or pediocin (De Alba, Bravo, & Medina, 2013; Hereu et al., 2012), enterocins A or B and sakacin A (Jofré, Aymerich, Monfort, & Garriga, 2008) has been assayed in different products. However, to our knowledge, the application of HHP with the LP system or LF on sliced dry-cured ham has not been investigated.

The aim of this work was to investigate the effect of HHP, applied individually or in combination with the LP system or LF on sliced dry-cured ham inoculated with *L. monocytogenes* and *S.* Enteritidis and to evaluate the changes in total viable counts (TVC), the physicochemical, rheological and color characteristics of the product stored under mild temperature abuse conditions (8 ºC) during 60 d.

2. Materials and methods

2.1. Microorganisms

L. monocytogenes INIA H66a from the INIA Culture Collection, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Madrid, Spain) and a mixture of *S.* Enteritidis strains CECT 4155, CECT 4300, and CECT 4396 from the Spanish Type Culture Collection (Valencia, Spain) were kept frozen at -80 ºC in tryptic soy yeast extract broth (TSYEB) (Biolife, Milano, Italy) with 30% glycerol. Samples were inoculated with over-night TSYEB cultures at 37 ºC for 18 h.

2.2. Sample preparation

Dry-cured ham was obtained from a local market in Madrid (Spain). Samples (20 g) aseptically sliced were inoculated by spreading *L. monocytogenes* and *Salmonella* cultures on the surface to achieve a final population of approximately 10^5 cfu/g. Non-inoculated samples were also prepared to determine total viable counts (TVC), pH, a_w, texture and color.

2.3. Preparation and addition of biopreservatives

Lactoperoxidase from bovine milk (DMV International, Veghel, Netherlands) was prepared in distilled-deionized water (15 mg/mL), sterilized by passing through a 0.2 µm-pore-size cellulose acetate filters (Millipore, Bedford, MA) and stored at -40 ºC. LP activity was quantified before addition to sliced dry-cured ham according to the method of Marshall, Cole, and Bramley (1986) by determination of the oxidation of ABTS (2,2´-azino-bis-[3-ethylbenzthiazoline-sulphonic acid]) (Sigma-Aldrich, Alcobendas, Spain) at 412 nm and expressed in ABTS units according to Shindler, Childs, and Bardsley (1976). Potassium thyocianate (KSCN, Merck, Darmstadt, Germany) was prepared in a 1 M aqueous solution and autoclaved at 121 ºC for 15 min. Glucose (Panreac, Barcelona, Spain) stock (1 M) and Glucose oxidase (Sigma-Aldrich, Alcobendas, Spain) stock at a concentration of 2260 U/mL were prepared

according to Kennedy et al. (2000). Glucose and GOX were sterilized by means of 0.2 µm-pore-size cellulose acetate filters and stored at room temperature and at 4 ºC, respectively, until use. The LP system was activated *in vitro* in sterilized eppendorf tubes by adding LP to reach an estimated final activity of 2.8 ABTS U/g followed by 0.03 mg/g of KSCN, 0.16 mg/g of glucose and 0.15 U/g of GOX. The activated LP system was added immediately after to each sample.

Bovine lactoferrin partially (15 to 20%) iron saturated according to the manufacturer, was obtained from DMV International. A 40 mg/mL solution of LF in distilled-deionized water was used as the stock solution for assay, sterilized by means of 0.2 µm-pore-size cellulose acetate filters and stored at -20 ºC until use. Bovine LF was added to each sample at a concentration of 1 mg/g.

2.4. HHP processing

Inoculated and non-inoculated slices were individually vacuum-packaged in double bags of BB325 (Oxygen permeability 25 cm^3/m^2; Cryovac Grace S.A., Barcelona, Spain). Samples were pressurized at 450 MPa and one holding time (10 min) at approx. 12 ºC. HHP treatments were performed in a high-pressure prototype ACIP 6000 (ACB, Nantes, France) of 3.5-L capacity and 600 MPa maximum working pressure. Water was used as a pressure transmitting medium.

Experimental treatments were LP system, LF, 450 MPa for 10 min, 450 MPa for 10 min combined with LP system, 450 MPa for 10 min combined with LF, and non-pressurized control group. After pressurization, treated and non-treated samples were stored at 8 ºC during 60 d. Two independent trials were performed on two different days.

2.3. Microbiological analysis

Samples were analyzed immediately after HHP treatments (time 0) and after 1, 15, 30 and 60 d of storage. Samples (20 g) were homogenized in 180 mL of 0.1% (wt/vol) sterile peptone water using a homogenizer (IUL, Barcelona, Spain). Decimal

dilutions of the homogenate were prepared in the same sterile solution. *Listeria* and *Salmonella* populations were determined on duplicate plates of Chromagar Listeria (Oxoid Ltd., Basingstoke, England) and Salmonella Shigella agar (SSA) (Scharlab S.L., Barcelona, Spain), incubated at 37 ºC for 48 and 24 h, respectively. If necessary, an initial enrichment step of *Salmonella* samples was included by adding the samples (20 g) to 180 mL of 0.1% sterile peptone water and incubated at 37 ºC for 24 h. This was followed by a secondary enrichment where 100 µL of the primary enrichment was added to 9 mL Rappaport-Vassiliadis broth (Oxoid Ltd). After incubation at 37 ºC for 24 h, enrichment broth was streaked on duplicate plates of SSA which were examined after incubation at 37 ºC for 24 h to detect presence or absence of *Salmonella*. TVC were determined on duplicate plates of tryptic soy agar (TSA) (Biolife) and incubated at 30 ºC for 72 h.

2.4. pH and a_w

pH determinations were taken using a pH-meter (model GPL22, Crison Instruments, Barcelona, Spain). Samples (10 g) of dry-cured ham were homogenized in 90 mL of distilled water in stomacher bags for 90 s. Water activity (a_w) was measured using the AquaLab Series 3 equipment (Decagon Devices, Inc., Pullman, WA, EE.UU.). Samples were taken 1, 30 and 60 d after treatments. Two measurements were performed per sample.

2.5. Textural determinations

Sliced dry-cured ham texture was determined using an Instron Compression Tester 4301 (Instron Ltd., Barcelona, Spain) controlled by the BlueHill V2.0 software, with a load cell of 1000 N and crosshead speed of 100 mm/min. The shear strenght (N) required to shear the sample was examined using a Warner-Bratzler blade and hardness (N), considered as the maximum force required to compress the sample was determined with a Kramer cell. Three measurements were taken per sample in dry-cured ham 1, 30 and 60 d after treatments.

2.6. Color measurements

Surface color of sliced dry-cured ham was measured twelve times per sample using a Minolta CM 700d Chromometer (Minolta Camera Co., Osaka, Japan). *L** (lightness, intensity of white color), *a** (+a, red;-a, green) and *b** (+b, yellow; -b, blue) were determined 1, 30 and 60 d after treatments.

2.7. Statistical analysis

Data were subjected to analysis of variance (ANOVA) by means of SPSS program Win 12.0 software (SPSS Inc., Chicago, IL, EE.UU.), with treatment and time of storage as main effects. Significant differences between means were assessed by Tukey test with a confidence interval of 95%.

3. Results and Discussion

3.1. Inactivation of L. monocytogenes *and* S. *Enteritidis*

Counts of *L. monocytogenes* in dry-cured ham subjected to LP system, LF, HHP or their combinations and stored at 8 ºC are shown in Table 1. According to the analysis of variance, treatments and storage time had significant ($P<0.001$) effect on *Listeria* counts. *L. monocytogenes* mean initial counts in inoculated dry-cured ham were 5.27 log cfu/g. Immediately after pressurization, reductions of *L. monocytogenes* were 0.58 log cfu/g in pressurized ham at 450 MPa for 10 min. Counts of *L. monocytogenes* were not affected by the LP system or LF applied individually. The reductions in *L. monocytogenes* counts in pressurized samples at 450 MPa combined with the LP system or LF were 0.58 and 0.55 log cfu/g, respectively. Immediately after pressurization, these combinations did not provided an additional advantage over use of the pressure treatment applied individually.

During the storage under refrigeration at 8 ºC, counts of *L. monocytogenes* only diminished by 0.4 log cfu/g in non-treated samples, indicating the ability of *L. monocytogenes* to survive in dry cured ham during prolonged storage. At the end of

the storage period, counts of *L. monocytogenes* in pressurized samples at 450 MPa were 0.64 log cfu/g lower than in non-treated samples. The reductions in *L. monocytogenes* counts in pressurized samples at 450 MPa combined with the LP system or LF were 0.56 and 0.86 log cfu/g, respectively. The inactivation achieved at 450 MPa combined with LP system was 0.26 log units higher than the sum of log reductions obtained with each single treatment and as a result, a slight synergistic antimicrobial effect was observed. On the contrary, pressurization combined with LF did not increase the antimicrobial activity against the pathogen. The inactivation of *L. monocytogenes* after treatment at 450 MPa for 10 min in Serrano ham was evaluated by Morales, Calzada, and Nuñez, (2006). They observed that HHP treatment reduced *L. monocytogenes* Scott A populations in 1.16 log cfu/g. In the present work, reductions of *L. monocytogenes* INIA H66a were lower than detected by Morales et al. (2006), confirming the reported variability among *L. monocytogenes* strains in response to HHP processing (Murano, Murano, Brennan, Shenoy, & Moreira, 1999; Tay, Shellhammer, Yousef, & Chism, 2003).

Counts of *S. Enteritidis* in dry-cured ham subjected to the LP system, LF, HHP or their combinations and stored at 8 ºC are shown in Table 2. Analysis of variance revealed a significant influence of treatments and storage time ($P<0.001$). *S*. Enteritidis mean initial counts in inoculated dry-cured ham were 4.67 log cfu/g. Immediately after pressurization, reductions of *S*. Enteritidis were 2.22 log cfu/g in pressurized ham at 450 MPa for 10 min. *Salmonella* counts were not affected by LP system or LF, applied individually. The reductions in *S*. Enteritidis counts in pressurized samples at 450 MPa combined with the LP system or LF were 4.32 and 3.75 log cfu/g, respectively. The inactivation achieved at 450 MPa combined with the LP system or LF was 2.10 and 1.53 log units higher than the sum of log reductions obtained with each single treatment, respectively. Therefore, a synergistic antimicrobial effect was detected on *S*. Enteritidis counts with combined treatments.

During the storage under refrigeration at 8 ºC, counts of *S. Enteritidis* diminished by 1.94 log cfu/g in non-treated samples. This decrease was also

observed in a previous work by De Alba et al. (2012a), indicating that *Salmonella* cells were affected by the adverse conditions of this meat product. From 1 d after treatments to the end of storage at 8 ºC, *Salmonella* was not detected in samples treated with pressure and the LP system, neither from 15 d in pressurized samples treated with LF. Damaged cells were not able to repair during 60 d at 8 ºC and *Salmonella* was only detected after enrichment of samples. At the end of the storage period, in samples where the pressurization was applied individually or in combination with antimicrobials, the pathogen counts were below the detection limit but their presence was recorded. These results reflected the inability of *Salmonella* to repair sublethal damage that occurs on exposure to high pressure treatment or to its combination with antimicrobials.

The effect of the LP system on the growth of *Escherichia coli* O157:H7, *L. monocytogenes* and *Staphylococcus aureus* has been evaluated in a ground beef model by Kennedy et al. (2000). As the antimicrobial activity occurs during bacterial growth, the effects of the treatment were most pronounced against bacterial species able to grow at the temperatures used during and subsequent to the LP system treatment. They observed that the effects were greater against *L. monocytogenes* which was able to grow at low temperatures, than against *E. coli* O157:H7 which survived but did not grow readily under those conditions. In all cases, the ability of inhibition was highly temperature dependent. Elliot et al. (2004) studied the effect of the LP system on the growth of foodborne pathogens in an experimental beef cube system at 37 ºC, 12 ºC and during a chilling regime (12 to -1 ºC). They suggested that the application of the LP system to meat provided an additional hurdle that prevented proliferation of the pathogenic bacteria at growth permissive temperature (12 ºC) and reduction of the pathogens at low temperatures not permissive for growth without compromising beneficial non pathogenic native bacteria. The effect of the LP system against *L. monocytogenes* in fresh meat was bacteriostatic (Kennedy et al., 2000; Elliot et al., 2004). The LP system combined with monolaurin in ground beef (McLay et al., 2002) failed to enhance inhibition of *E. coli* O157:H7 over use of

the LP system, applied individually at 37 ºC. Kennedy et al. (2000) noted that a reduced incubation temperature markedly increased the inhibition of *E. coli* O157:H7 by the LP system in ground meat and suggested that the metabolic state of the cell was significant in terms of effectiveness of this agent. A synergistic antimicrobial effect of HHP at 450 MPa for 10 min combined with the LP system was observed by Montiel et al. (2012) in cold-smoked salmon inoculated with *L. monocytogenes* and stored during 35 d at 5 ºC, avoiding the pathogen recovery.

The bactericidal activity of LF against some spoilage and pathogenic foodborne bacteria applied individually or in combination with HHP has been evaluated in some meat products. Del Olmo et al. (2009) studied the effect of LF against *Pseudomonas fluorescens* in ground meat and in meat fractions. The bactericidal activity of LF at 1 mg/g disappeared when it was added to ground meat held for 24 h at 5 ºC, different from what happened in distilled water after 1 h at 30 ºC, where reductions of 1.9 log units were achieved. This fact demonstrated that meat components could be responsible for the loss of antimicrobial activity. Those results are in agreement with the results obtained in the present work, where LF applied individually did not show activity against *Listeria* neither *Salmonella* in sliced dry-cured ham. Other authors have observed weak bactericidal activity or loss of activity of LF or lactoferricin in complex systems or foods (Del Olmo et al., 2008; Bellamy et al., 1993; Branen & Davidson, 2004; Branen & Davidson, 2000; Murdock & Matthews, 2002; Payne, Davidson, Oliver, & Christen, 1990; Payne, Oliver, & Davidson, 1994; Venkitanarayanan, Zhao, & Doyle, 1999; Chantaysakorn & Richter, 2000). Kang, Lee, and Chin, (2008) reported a slight bactericidal effect of ALF (activated lactoferrin) against *L. monocytogenes* when used in low-fat sausages and Heller et al. (2007) reported no enhancement in *E. coli* contamination on beef cuts by ALF in comparison with conventional treatments such as hot water or warm lactic acid rinse. According to Colak, Hampikyan, Bingol, and Aksu, (2008), *E. coli* and *Pseudomonas* spp. inoculated in Turkish meatballs were reduced significantly by LF alone or in combination with nisin. The combined treatment between nisin (200 µg/g) and LF

(100 µg/g) had a maximum effect on *S. aureus* in meatball samples but LF alone was ineffective at inhibiting its growth.

Masschalck, Van Houdt, and Michiels (2001) observed that the bactericidal effect of LF combined with HHP was enhanced under *in vitro* conditions. In chicken filets, 400 MPa for 10 min combined with LF slightly improved the bactericidal effect of individual treatments on *P. fluorescens*, *E. coli* O157:H7 and *L. monocytogenes* during 9 d at 5 ºC (Del Olmo et al., 2012a, b). A synergistic effect was observed in the present work with the combination of HHP and LF against *Salmonella* in sliced dry-cured ham, showing a higher sensitivity to the combined treatment during 60 d at 8 ºC. This fact seems to confirm that factors in foodstuffs such as water activity, pH, temperature, food components such as lipids, proteins, carbohydrates, presence of cations, and microbial population can influence the effectiveness of LF and its derivatives but further studies would be necessary to understand the underlying mechanisms.

3.2. Inactivation of total viable counts (TVC)

TVC in dry-cured ham treated with LPS, LF, HHP or their combinations and stored at 8 ºC during 60 d are given in Table 3. Statistically significant ($P<0.001$) effect of treatments and storage time were detected. Initial TVC in non-treated dry-cured ham were 3.59 log cfu/g. Pressurization reduced TVC by 1.52 log cfu/g and reductions of 1.41 and 1.48 log cfu/g were achieved by combined treatments with the LP system or LF, respectively, one day after treatments. An increase of TVC was observed in non-treated and treated samples with antimicrobials, applied individually, during 60 d at 8 ºC. Pressurized samples and those treated with HHP and LF exhibited a rising trend of TVC from 30 d until the end of storage period because of microbiota recovery. Only samples treated at 450 MPa combined with the LP system showed TVC levels significantly ($P<0.05$) lower than non-treated samples, achieving reductions of 2.19 log cfu/g in sliced dry-cured ham at the end of storage. As a result, a clear synergistic effect was observed because the reductions obtained with

the combined treatment were 2.09 log units higher than the sum of the inactivation obtained with each single treatment.

The growth of most microbial populations in a ground beef model was strongly inhibited in the presence of the LP system (Kennedy et al., 2000). On the contrary, in the present work, the LP system applied individually did not provide an additional benefit in sliced dry-cured ham compared with non-treated samples. It is known that the effect of the LP system depends on bacterial species that are present and the medium conditions, such as pH and storage temperature of food (Seifu et al., 2005). Elliot et al. (2004) examined the effect of the LP system on beef microbiota and they observed that at chilling temperatures, the LP system inhibited the growth of pseudomonads but did not prevent the development of lactic acid bacteria, although their growth was retarded during 42 d of refrigeration. The combination between HHP at 450 MPa for 10 min and the LP system inactivated TVC in smoked salmon, remaining under detectable levels during 35 d of refrigeration at 5 ºC (Montiel et al., 2012). Significant reductions in total aerobic bacteria, coliforms, total psychrophilic bacteria, yeasts and molds levels were reported in Turkish-style meatballs (Colak et al., 2008), achieving the largest reductions with a mixture of LF (200 µg/g) and nisin (100 µg/g). This combination extended the refrigerated shelf-life of this product to 10 days.

3.2. pH and a_w

Values of pH and a_w in dry-cured ham are shown in Table 4. Analysis of variance revealed a significant influence of treatments ($P<0.05$) and storage time ($P<0.001$) on pH values. Slight increase ($P<0.05$) in pH was observed in samples treated with LF, one day after treatments. The opposite effect was observed in samples treated with combined treatments, showing a decrease ($P<0.05$) in pH values. No significant differences between non-treated samples and those treated with either the LP system or HHP were observed. After 30 d under storage at 8 ºC, a significant increase ($P<0.05$) of pH was observed in all samples. However, values of pH tended to

attenuate in the product after 60 d at 8 ºC in accordance with De Alba et al. (2012a), although those pH values were higher at the end of storage than at one day after treatments. Colak et al. (2008) observed that pH levels of Turkish meatballs increased during storage time for all treated (LF, nisin, and their combinations) and non-treated groups. However, the pH of Turkish meatballs was not significantly affected by the addition of different LF and nisin concentrations.

Treatments and storage time had a significant ($P<0.001$) effect on a_w values. Values of a_w increased significantly ($P<0.05$) owing to treatments, although tended to stabilize in the product at the end of the stored period at 8 ºC. Minimal a_w values for the growth of *Salmonella* of approximately 0.95 (Sperber, 1983) has been estimated. In sliced dry-cured ham used in this work, with values of a_w between 0.865 and 0.879, recovery of sublethally injured cells would be impeded by the lower a_w in the product. Dry-cured ham shows a low a_w (usually lower than 0.92) and high salt concentration (higher than 4%). In case of a potential contamination, these intrinsic characteristics hardly support the growth of *L. monocytogenes* but may allow *L. monocytogenes* survival (ICMSF, 1996). These findings are in accordance with our results because *L. monocytogenes* did not grow but however, it was able to survive in dry-cured ham stored during 60 d at 8 ºC.

3.3. Texture

Textural properties of sliced dry-cured ham stored during 60 d at 8 ºC are shown in Table 5. No statistically significant effect of treatments and time of storage was detected on shear force. No significant differences in shear strength values (N) were observed one day after treatments. Samples treated with HHP and the LP system showed an increase ($P<0.05$) of shear strength values at the end of the refrigerated period. According to the analysis of variance, hardness measured as maximum force (N) was only significantly ($P<0.05$) affected by time of storage. One day after treatments, hardness increased in samples treated with LF compared with non-treated samples, but this rising was lower for the rest of treatments. Differences

were attenuated at the end of storage. One possible explanation for differences observed in the present work could be the heterogeneity of samples.

HHP treatment at 450 MPa for 10 min has no effect on the sensory characteristics of sliced dry-cured Serrano ham determined 7 d after pressurization (Morales et al., 2006). In smoked salmon, hardness and shear strength values tended to increase when HHP treatments at 450 MPa for 10 min were applied alone or in combination with the LP system (Montiel et al., 2012). Further studies about the effect of combined treatments between HHP, the LP system or LF should be carried out in different meat products to evaluate possible changes on textural properties.

3.4. Color

The effect of treatments on color (CIE *L**, *a**, *b**) of vacuum-packaged sliced dry-cured ham is shown in Table 6. Analysis of variance revealed a significant influence of treatments ($P<0.001$) on *L**, *a** and *b**. *L** decreased slightly ($P<0.05$) in samples treated with the LP system applied individually, although the differences with non-treated samples were very light one day after treatments. No significant differences between samples treated with LF or pressurized at 450 MPa were observed. However, combined treatments between HHP and the LP system or LF showed an increase ($P<0.05$) of *L** compared with non-treated samples. These changes were attenuated at the end of the refrigerated storage at 8 ºC.

No significant differences in *a** were observed one day after treatments. Samples treated with pressure, applied individually or in combination with antimicrobials, showed a decrease ($P<0.05$) of *a** at the end of storage. On the other hand, antimicrobials applied individually did not affect *a** values. One day after treatments, samples treated with HHP and LF showed an increase of *b** values regarding non-treated samples, but these differences were attenuated at the end of storage.

No significant effect of pressurization at 450 MPa for 10 min on *L**, *a** and *b** values was observed by Morales et al. (2006) in Serrano ham, although differences in *L** and *a** values between different points on the same slice were found. The LP system

has been reported to have little effect on the sensory and physical characteristics of treated milk and dairy products (Martinez, Mendoza, Alacron, & García, 1988; Ridley, & Shalo, 1990) but to our knowledge, a few studies have been published about the effect of this antimicrobial system on meat products. Tan and Ockerman (2008) evaluated the effect of the LP system on physical and sensory characteristics of marinated broiler drumsticks. Treated samples showed lower skin and muscle *a** values, when compared with control samples. According to these authors, this reduction of *a** values of the LP system-treated samples could be probably due to the addition of H_2O_2 used to activate the system, a strong oxidizing agent that is occasionally used as a bleaching agent in the food industry (Daeschel & Penner, 1992). *L**, *a** and *b** values also were affected by HHP and the LP system in smoked salmon (Montiel et al., 2012) with an increase in *L**, whereas the impact on redness (*a**) and yellowness (*b**) was lower. In the present work, *a** values were not affected by the LP system, one day after treatments. The generation *in situ* of the hydrogen peroxide by added glucose oxidase (GOX) and glucose did not affect negatively to color characteristics of dry-cured ham.

4. Conclusions

The antimicrobial effect of combined treatments of 450 MPa for 10 min and the LP system on *L. monocytogenes* in sliced dry-cured ham was slightly enhanced. Higher inactivation was achieved on *S.* Enteritidis with a synergistic antimicrobial effect of pressurization combined with the LP system or LF. The biopreservatives LP system or LF applied individually did not affect pathogen counts. A clear synergistic antimicrobial effect was detected on TVC levels with HHP combined with the LP system at the end of storage. Pressurization combined with biopreservatives modified slightly aspects of quality of dry-cured ham, such as texture and color. Further studies about the use of the LP system, LF and their combinations with HHP on meat

products to improve their microbiological safety and to determine possible changes on physicochemical and rheological characteristics merit investigation.

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Table 1. *L. monocytogenes* counts (log cfu/g) in dry-cured ham treated with HHP at 450 MPa, the LP system, LF and their combinations, during 60 d at 8 ºC.

	0 h	1 d	15 d	30 d	60 d
Control	5.27 ± 0.12 ^{cC}	5.34 ± 0.09 ^{cC}	5.25 ± 0.14 ^{cBC}	5.14 ± 0.14 ^{dB}	4.87 ± 0.31 ^{cA}
LPS	5.07 ± 0.35 ^{bB}	5.20 ± 0.10 ^{bcC}	5.11 ± 0.17 ^{bBC}	5.10 ± 0.12 ^{dBC}	4.91 ± 0.13 ^{cA}
LF	5.39 ± 0.11 ^{dBC}	5.47 ± 0.43 ^{cC}	5.31 ± 0.23 ^{cABC}	5.13 ± 0.19 ^{dAB}	5.02 ± 0.09 ^{dA}
450 MPa	4.69 ± 0.12 ^{aC}	4.90 ± 0.18 ^{abD}	4.41 ± 0.09 ^{aB}	4.34 ± 0.33 ^{cB}	4.23 ± 0.18 ^{bA}
450 MPa + LPS	4.72 ± 0.12 ^{aD}	4.91 ± 0.10 ^{abE}	4.51 ± 0.12 ^{aC}	4.15 ± 0.36 ^{aB}	4.01 ± 0.28 ^{aA}
450 MPa + LF	4.69 ± 0.15 ^{aD}	4.70 ± 0.22 ^{aD}	4.49 ± 0.09 ^{aC}	4.26 ± 0.42 ^{bA}	4.31 ± 0.12 ^{bB}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 2. *S.* Enteritidis counts (log cfu/g) in dry-cured ham treated with HHP at 450 MPa, the LP system, LF, and their combinations, during 60 d at 8 ºC.

	0 h	1 d	15 d	30 d	60 d
Control	4.67 ± 0.69 ^{dA}	4.71 ± 0.58 ^{dA}	3.87 ± 1.83 ^{bA}	3.12 ± 2.05 ^{bA}	2.73 ± 1.87 ^{bA}
LPS	4.66 ± 0.31 ^{dB}	4.79 ± 0.31 ^{dB}	4.41 ± 0.53 ^{bB}	3.47 ± 1.59 ^{bAB}	2.64 ± 1.86 ^{bA}
LF	4.59 ± 0.64 ^{dBC}	4.68 ± 0.36 ^{dC}	4.53 ± 0.50 ^{bBC}	2.89 ± 1.88 ^{bAB}	2.61 ± 1.41 ^{bA}
450 MPa	2.45 ± 0.60 ^{cD}	1.35 ± 0.44 ^{cC}	0.60 ± 0.24 ^{aB}	0.08 ± 0.14 ^{aA}	PRE ^{aA}
450 MPa + LPS	0.35 ± 0.37 ^{aB}	PRE ^{aA}	PRE ^{aA}	PRE ^{aA}	PRE ^{aA}
450 MPa + LF	0.92 ± 0.08 ^{bC}	0.25 ± 0.31 ^{bB}	PRE ^{aA}	PRE ^{aA}	PRE ^{aA}

PRE, presence in 20 g. Limit of detection 10 cfu/g.
Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 3. Levels (log cfu/g) of total viable counts (TVC) in dry-cured ham treated with HHP at 450 MPa, the LP system, LF, and their combinations, during 60 d at 8 ºC.

	1 d	30 d	60 d
Control	3.59 ± 0.19 ^{bA}	4.22 ± 0.26 ^{bB}	5.07 ± 0.24 ^{bcC}
LPS	3.40 ± 0.46 ^{bA}	4.21 ± 0.14 ^{bB}	5.21 ± 1.05 ^{dC}
LF	3.16 ± 1.28 ^{bA}	4.48 ± 0.22 ^{bB}	5.17 ± 0.28 ^{cdB}
450 MPa	2.07 ± 0.34 ^{aA}	3.00 ± 0.65 ^{aB}	5.03 ± 0.24 ^{bC}
450 MPa + LPS	2.18 ± 0.33 ^{aA}	3.18 ± 0.61 ^{aB}	2.88 ± 0.56 ^{aB}
450 MPa + LF	2.11 ± 0.35 ^{aA}	2.83 ± 1.21 ^{aB}	4.95 ± 0.39 ^{bC}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 4. Values of pH and a_w in sliced dry-cured ham treated with HHP at 450 MPa, the LP system, LF, and their combinations, during 60 d at 8 ºC.

		1 d	30 d	60 d
pH	Control	5.84 ± 0.05 ^{bA}	6.08 ± 0.10 ^{aB}	6.05 ± 0.12 ^{bB}
	LPS	5.84 ± 0.04 ^{bA}	6.33 ± 0.34 ^{aB}	5.96 ± 0.07 ^{aAB}
	LF	5.93 ± 0.08 ^{cA}	6.07 ± 0.09 ^{aB}	5.96 ± 0.05 ^{aA}
	450 MPa	5.88 ± 0.08 ^{bA}	6.24 ± 0.07 ^{aC}	6.12 ± 0.06 ^{cB}
	450 MPa + LPS	5.69 ± 0.08 ^{aA}	6.25 ± 0.12 ^{aC}	6.00 ± 0.12 ^{abB}
	450 MPa + LF	5.68 ± 0.03 ^{aA}	6.14 ± 0.06 ^{aC}	6.00 ± 0.04 ^{abB}
a_w	Control	0.865 ± 0.006 ^{aA}	0.866 ± 0.004 ^{aA}	0.870 ± 0.005 ^{abA}
	LPS	0.873 ± 0.004 ^{bA}	0.874 ± 0.002 ^{cA}	0.872 ± 0.005 ^{bA}
	LF	0.871 ± 0.003 ^{bB}	0.870 ± 0.002 ^{bB}	0.866 ± 0.008 ^{aA}
	450 MPa	0.876 ± 0.003 ^{cB}	0.874 ± 0.003 ^{cB}	0.869 ± 0.005 ^{abA}
	450 MPa + LPS	0.878 ± 0.001 ^{cB}	0.879 ± 0.004 ^{dB}	0.871 ± 0.003 ^{bA}
	450 MPa + LF	0.877 ± 0.002 ^{cC}	0.875 ± 0.002 ^{cB}	0.871 ± 0.005 ^{bA}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 5. Texture properties of sliced dry cured ham treated with HHP at 450 MPa, the LP system, LF, and their combinations, during 60 d at 8 ºC.

		1 d	30 d	60 d
Shear force (N)	Control	38.23 ± 10.56 ^{aA}	35.56 ± 10.82 ^{abA}	37.38 ± 9.09 ^{aA}
	LPS	35.70 ± 8.78 ^{aA}	30.56 ± 4.29 ^{aA}	32.71 ± 7.77 ^{aA}
	LF	36.99 ± 12.71 ^{aA}	36.53 ± 4.52 ^{abA}	37.21 ± 15.83 ^{aA}
	450 MPa	36.05± 6.90 ^{aA}	37.93 ± 6.00 ^{bA}	38.93 ± 10.25 ^{aA}
	450 MPa + LPS	30.14 ± 6.82 ^{aA}	36.73 ± 11.20 ^{abAB}	38.06 ± 4.76 ^{aB}
	450 MPa + LF	38.11 ± 16.12 ^{aA}	34.75 ± 14.28 ^{abA}	38.91 ± 12.83 ^{aA}
Hardness (N)	Control	405.88 ± 59.59 ^{aA}	428.80 ± 64.47 ^{aA}	441.22 ± 112.81 ^{aA}
	LPS	481.01 ± 47.56 ^{abA}	504.37 ± 163.14 ^{aA}	444.77 ± 113.26 ^{aA}
	LF	542.33 ± 91.76 ^{bB}	468.19 ± 85.04 ^{aAB}	374.50 ± 124.99 ^{aA}
	450 MPa	438.98 ± 155.72 ^{abA}	445.31 ± 60.48 ^{aA}	467.83 ± 136.76 ^{aA}
	450 MPa + LPS	458.09 ± 95.63 ^{abA}	504.76 ± 98.58 ^{aA}	453.68 ± 104.41 ^{aA}
	450 MPa + LF	467.23 ± 105.96 ^{abB}	449.80 ± 50.50 ^{aB}	348.86 ± 133.20 ^{aA}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 6. Color parameters of sliced dry cured ham treated with HHP at 450 MPa, the LP system, LF, and their combinations, during 60 d at 8 ºC.

		1 d	30 d	60 d
*L**	Control	40.05 ± 2.44 ^{abB}	38.78 ± 2.58 ^{abAB}	37.65 ± 3.29 ^{abA}
	LPS	39.32 ± 2.71 ^{aA}	39.60 ± 3.37 ^{abA}	38.05 ± 3.40 ^{abcA}
	LF	39.64 ± 3.15 ^{abB}	37.76 ± 3.67 ^{aA}	37.24 ± 3.07 ^{aA}
	450 MPa	40.38 ± 3.60 ^{abA}	40.77 ± 3.14 ^{bcA}	39.78 ± 3.23 ^{bcA}
	450 MPa + LPS	42.28 ± 2.30 ^{cB}	40.19 ± 3.84 ^{bcA}	39.69 ± 4.20 ^{bcA}
	450 MPa + LF	41.38 ± 2.53 ^{bcAB}	41.86 ± 1.82 ^{cB}	40.25 ± 3.39 ^{cA}
*a**	Control	14.57 ± 1.71 ^{aB}	13.24 ± 2.80 ^{aA}	15.42 ± 2.66 ^{cB}
	LPS	14.61 ± 1.48 ^{aA}	14.67 ± 2.03 ^{bA}	14.67 ± 3.15 ^{bcA}
	LF	14.87 ± 1.81 ^{aA}	14.12 ± 1.81 ^{abA}	15.85 ± 2.13 ^{cB}
	450 MPa	14.24 ± 1.71 ^{aB}	14.36 ± 2.27 ^{bB}	13.07 ± 2.65 ^{aA}
	450 MPa + LPS	14.70 ± 1.85 ^{aB}	15.00 ± 2.27 ^{bB}	13.30 ± 2.05 ^{abA}
	450 MPa + LF	15.09 ± 0.98 ^{aB}	14.98 ± 1.16 ^{bB}	13.73 ± 2.05 ^{abA}
*b**	Control	8.85 ± 1.50 ^{abA}	12.72 ± 3.19 ^{bB}	8.63 ± 3.31 ^{abA}
	LPS	8.51 ± 2.11 ^{abA}	14.74 ± 2.53 ^{cB}	7.95 ± 3.02 ^{aA}
	LF	8.18 ± 1.81 ^{aA}	13.18 ± 2.69 ^{bcC}	9.83 ± 2.63 ^{bB}
	450 MPa	8.12 ± 1.67 ^{aA}	10.45 ± 2.75 ^{aB}	7.91 ± 3.31 ^{aA}
	450 MPa + LPS	9.15 ± 1.57 ^{abB}	10.29 ± 2.57 ^{aB}	7.61 ± 2.57 ^{aA}
	450 MPa + LF	9.45 ± 1.52 ^{bA}	10.92 ± 1.55 ^{aB}	8.32 ± 3.05 ^{abA}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Highlights

- The antimicrobial effect of HHP at 450 MPa for 10 min combined with the LP system or LF on *Salmonella* Enteritidis in sliced vacuum packed dry-cured ham was synergistic.

- The combination of HHP and the LP system on *Listeria monocytogenes* was only slightly synergistic.

- Textural and color parameters were slightly modified.

3.4. High pressure treatments on the inactivation of *Salmonella* Enteritidis and the characteristics of beef carpaccio*. Meat Science (2012) 92: 823-828.*

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High pressure treatments on the inactivation of *Salmonella* Enteritidis and the characteristics of beef carpaccio

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ABSTRACT

The effect of high pressure (HP) on *Salmonella enterica* subsp. *enterica* serovar Enteritidis in beef carpaccio stored under temperature abuse conditions (8 °C) during 30 days was investigated. After treatment, reductions of *S.* Enteritidis were 3.68 and 5.94 log cfu/g in samples pressurized at 450 MPa for 5 and 10 min, respectively, whereas the pathogen was only detected after enrichment of samples treated at 450 MPa for 15 min. During storage, counts of *S.* Enteritidis decreased 0.26 log cfu/g in non-pressurized carpaccio, 1.33 log cfu/g in carpaccio treated at 450 MPa for 5 min and were only detected after enrichment in carpaccio pressurized at 450 MPa for 10 or 15 min. Color (L^*, a^* and b^*) varied with pressurization and storage, with higher changes in carpaccio treated at longer pressurization times. Shear resistance was slightly lower in treated samples just after pressurization, but increased at the end of the storage period. Maximum force was less affected by treatment.

1. Introduction

Beef carpaccio is a ready to eat meat product (RTE) consumed without further treatment. Thinly sliced fillets of beef are prepared from raw or cured pieces of meat, which are frozen, sliced, packaged under vacuum or modified atmosphere without oxygen and marketed at refrigeration temperatures. Raw beef can become contaminated during slaughter. Hazards associated with raw beef products include *Salmonella* spp., *Escherichia coli* O157:H7 and *Listeria monocytogenes* (FDA, 2009). Beef carpaccio can also be contaminated with pathogens during its preparation and storage. Carpaccio should be considered a high-risk food product even at moderate levels of contamination.

Salmonella has long been recognized as an important zoonotic pathogen of economic significance in animal and humans. *Salmonella* is found in the environment and in the gastrointestinal tract of farmed and wild animals. The majority of human cases are linked to contaminated foods. Safe handling of raw meat and other raw food ingredients, through cooking and good kitchen hygiene can prevent or reduce the risk posed by contaminated food. A total of 108,614 confirmed cases of Salmonellosis were reported in the European Union in 2009, although cases attributed to *S.* Enteritidis have decreased recently (EFSA, 2012). However, *Salmonella* infections have not declined over the past 15 years in the United States (CDC, 2011), being an important cause of human illness. *Salmonella* has been found in retail raw beef, steak tartare and pork products (Busani et al., 2005; Duffy et al., 2001; Kivi et al., 2007; Sorensen et al., 2002; Zhao, Doyle, Fedorka-Cray, Zhao, & Ladely, 2002; Zhao et al., 2001), although the incidence reported was low (1.0 to 9.6%). Beef carpaccio was involved in a Salmonellosis outbreak in Denmark (Ethelberg et al., 2007).

High pressure (HP) treatment is a non-thermal technology used to improve the safety and extend the shelf-life of food products. Industrial application of high pressure treatments has increased rapidly, mainly in RTE meat products. Numerous studies deal with the sensitivity to pressure of various microorganisms naturally present or inoculated in meat and meat products such as minced meat (Carlez, Rosec, Richard, & Cheftel, 1994), chicken breast fillets (Morales, Calzada, Rodriguez, De Paz, & Nuñez, 2009), fermented sausages (Marcos, Aymerich, & Garriga, 2005), cured pork carpaccio (Realini, Guàrdia, Garriga, Pérez-Juan, & Arnau, 2011), sliced vacuum-packaged dry-cured ham (De Alba, Montiel, Bravo, Gaya, & Medina, 2012) and others. The effect of HP on foodborne pathogens and factors affecting microbial resistance has been reviewed by Rendueles et al. (2011). However, pressurization causes undesirable changes in raw meat color, with a whitening effect and important decreases in redness (Carlez, Veciana-Nogués, & Cheftel, 1995), mainly related with the pressure intensity. Although many studies have been conducted on the use of high pressure in different meat products, to our knowledge, the application of this technology on carpaccio inoculated with *Salmonella* spp. and the behaviour of the pathogen during storage has not been investigated. Storage under conditions of mild temperature abuse (8 °C) was selected as a temperature frequently found in domestic refrigerators (Garrido, García-Jalón, & Vitas, 2010).

The aim of this work was to evaluate the effect of high-pressure treatments on the survival of inoculated *Salmonella enterica* subsp.

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enterica ser. Enteritidis and the physicochemical, rheological and color characteristics of beef carpaccio stored for 30 days at 8 °C.

2. Materials and methods

2.1. Microorganisms

Salmonella Enteritidis strains CECT 4155, CECT 4300 and CECT 4396 from the Spanish Type Culture Collection (Valencia, Spain) were kept at −80 º C in tryptic soy yeast extract broth (TSYEB) (Biolife, Milano, Italy) with 30% glycerol. A three-strain cocktail of *S.* Enteritidis was prepared by mixing equal amounts of cultures of *Salmonella* strains, separately grown in TSYEB for 18 h at 37 °C.

2.2. Sample preparation and HP treatments

Beef carpaccio was obtained from a local market in Madrid (Spain). Samples (10 g) were aseptically prepared and inoculated by spreading the cocktail of *Salmonella* strains on the surface in order to achieve a final population of approximately 10^6 cfu/g. Control and inoculated samples were individually vacuum-packed in double bags of BB325 (Oxygen permeability 25 cm^3/m^2; Cryovac Grace S.A., Barcelona, Spain). Non-inoculated samples were also prepared to determine total viable counts (TVC), pH, a_w, texture and color.

High-pressure treatments were performed in a high-pressure prototype ACIP 6000 (ACB, Nantes, France) of 3.5-L capacity and 600 MPa maximum working pressure. Duplicated samples were pressurized at 450 MPa and three holding times (5, 10 and 15 min) respectively, at 12 °C giving three experimental treatments (450 MPa for 5, 10 and 15 min), as well as non-pressurized control group. Pressure come up time was 2.9 min. Water was used as pressure transmitting medium. After treatments, pressurized and non-pressurized samples were stored at 8 °C during 30 days. Two independent trials were carried out.

2.3. Microbiological analysis

Salmonella counts were determined after treatment (0 h) and at 1, 3, 7, 15, 21 and 30 days of storage. Non inoculated samples were analyzed for total viable counts (TVC) at 0 h and 15 and 30 days. Samples (10 g) were homogenized with 90 mL of sterile 0.1% (wt/vol) peptone water solution in stomacher bags for 90 s using a homogenizer (IUL, Barcelona, Spain). Decimal dilutions of the homogenate were prepared in the same sterile solution and the *Salmonella* population was determined on duplicate plates of Salmonella Shigella agar (SSA) (Scharlab S.L., Barcelona, Spain) incubated at 37 °C for 24 h. If necessary, an initial enrichment step was included by adding the samples (10 g) to 90 mL of 0.1% sterile peptone water and incubated at 37 °C for 24 h. This was followed by a secondary enrichment where 100 μL of the primary enrichment was added to 9 mL Rappaport-Vassiliadis broth (Oxoid Ltd., Basingstoke, England). After incubation at 37 °C for 24 h, enrichment broth was streaked on SSA duplicate plates, which were examined after incubation at 37 °C for 24 h to detect the presence or absence of *Salmonella*. TVC were determined on duplicate plates of tryptic soy agar (TSA) (Biolife) incubated for 72 h at 30 °C.

2.4. pH and a_w

Beef carpaccio samples (10 g) were homogenized with 90 mL of distilled water in stomacher bags for 90 s and pH determinations were taken using a pH-meter (model GPL 22, Crison Instruments, Barcelona, Spain). Water activity (a_w) was measured in the carpaccio using the AquaLab Series 3 equipment (Decagon Devices, Inc., Pullman, WA, USA). Samples were taken 1, 15 and 30 days after treatments. Two measurements were carried out for each sample.

2.5. Color measurements

Surface color of beef carpaccio was measured in different locations and the respective mean of six measurements per sample was expressed as the final value using a Minolta CM 700d Chromometer (Minolta Camera Co., Osaka, Japan). Before measurements, the chromometer was calibrated against the white tile supplied with the instrument. L^* (lightness), a^* (redness) and b^* (yellowness) were determined 1, 15 and 30 days after treatment for each sample.

2.6. Textural determinations

Texture parameters were measured using an Instron Compression Tester 4301 (Instron Ltd., Barcelona, Spain) controlled by the BlueHill V2.0 software, with a load cell of 1000 N and crosshead speed of 100 mm/min. The shear strength (N) required to shear through the sample was determined using a Warner-Bratzler blade and the maximum force (N) required to compress the sample with a Kramer cell. Four measurements were taken for each sample 1, 15 and 30 days after treatment.

2.7. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) by means of SPSS program Win 12.0 software (SPSS Inc., Chicago, IL), with treatment and time of storage as main effects. Significant differences between means were assessed by Tukey test with a confidence interval of 95 %.

3. Results and discussion

3.1. Effect of high pressure on Salmonella Enteritidis and total viable counts

Counts of *S.* Enteritidis in beef carpaccio subjected to 450 MPa for 5, 10 and 15 min and stored at 8 °C are shown in Table 1. High pressure treatment and storage time had a significant ($P<0.001$) effect on *Salmonella* counts. *S.* Enteritidis mean initial counts in inoculated beef carpaccio were 6.54 log cfu/g. After treatment, counts in SSA were 3.68 and 5.94 log cfu/g lower in samples pressurized at 450 MPa for 5 min and 10 min, respectively, than in control samples, whereas *Salmonella* was detected only after enrichment of samples treated at 450 MPa for 15 min (presence in 10 g). During the 30 days of storage at 8 °C, counts of *S.* Enteritidis decreased 0.26 log cfu/g in non-pressurized samples and 1.33 log cfu/g in samples treated at 450 MPa for 5 min. In carpaccio treated for 10 or 15 min, the pathogen did not recover, with levels below the detection limit by direct count

Table 1
Levels (log cfu/g) of *Salmonella* in pressurized beef carpaccio stored during 30 days at 8 °C.

Time	Non-pressurized	450 MPa/5 min	450 MPa/10 min	450 MPa/15 min
0 h	6.54 ± 0.08^{dCD}	2.86 ± 0.07^{cE}	0.60 ± 0.35^{bA}	PRE^{aA}
1 day	6.41 ± 0.03^{cB}	1.93 ± 0.15^{bD}	PRE^{aA}	PRE^{aA}
3 days	6.45 ± 0.08^{cBC}	3.44 ± 0.09^{bF}	PRE^{aA}	PRE^{aA}
7 days	6.55 ± 0.05^{cD}	2.00 ± 0.00^{bD}	PRE^{aA}	PRE^{aA}
15 days	6.45 ± 0.03^{cBCD}	1.45 ± 0.63^{bB}	PRE^{aA}	PRE^{aA}
21 days	6.29 ± 0.10^{cA}	1.08 ± 0.90^{bA}	PRE^{aA}	PRE^{aA}
30 days	6.28 ± 0.03^{cA}	1.53 ± 0.35^{bC}	PRE^{aA}	PRE^{aA}

Limit of detection 10 cfu/g.
PRE, presence in 10 g.
Values are means ± SD.
[a,b,c,d]Means within the same row with different superscripts differ significantly at $P<0.05$.
[A,B,C,D]Means within the same column with different superscripts differ significantly at $P<0.05$.

(<1 log cfu/g), although the presence of *S.* Enteritidis in 10 g samples was recorded.

High pressure processing at 600 MPa for 6 min inactivated both *Salmonella* spp. and *L. monocytogenes* present in sliced marinated beef loin during storage at 4 °C for 120 days (Garriga, Grébol, Aymerich, Monfort, & Hugas, 2004). In chicken breast fillets inoculated with *S.* Enteritidis, Morales et al. (2009) obtained 4.8 log cfu/g reductions with a 15 min treatment at 400 MPa. Other studies on the elimination of *Salmonella* by HP treatments in different meat products achieved reductions in *Salmonella* counts of approximately 2–3 log units in cooked ham pressurized at 400 MPa for 10 min (Aymerich, Jofré, Garriga, & Hugas, 2005). Lethality values for *S.* Enteritidis in dry-cured ham pressurized at 400, 500 or 600 MPa ranged from 1.1 to 4.3 log units (De Alba et al., 2012), lower than those attained in the present work. The microbial susceptibility to HP inactivation is affected by the food composition and physicochemical properties. Low water activity, as those found in cured ham, increase microbial resistance, decreasing the antimicrobial efficiency of pressurization (Patterson, 2005).

TVC in beef carpaccio subjected to high-pressure treatments at 450 MPa for 5, 10 and 15 min and stored at 8 °C are given in Table 2. Analysis of variance revealed a significant influence of treatment and time of refrigeration ($P<0.001$) on total microbiota. Initial TVC in non-pressurized beef carpaccio was 6.1 log cfu/g. Pressurization reduced TVC by 2.54, 3.12 and 3.43 log cfu/g in samples treated at 450 MPa for 5, 10 and 15 min, respectively. An increase of TVC (log cfu/g) was observed in all non-treated and treated samples during storage at 8 °C, although TVC (log cfu/g) in pressurized samples were at lower levels than in non-pressurized beef carpaccio. Differences in TVC after 30 days at 8 °C were 1.33, 0.68 and 2.67 log cfu/g in samples treated at 450 MPa for 5, 10 and 15 min, with respect to non-pressurized carpaccio samples.

Similar reductions of TVC at 400 and 600 MPa were observed by Realini et al. (2011) in cured pork carpaccio samples. Counts of lactic acid bacteria and psychrotrophs were close to 7 log cfu/g after 41 days in non pressurized cured pork carpaccio, whereas in pressurized samples levels were approx. 3 log units lower. HP treatments at 400 and 450 MPa in minced meat reduced TVC by 3 and 5 log units (Carlez et al., 1994). Other studies indicated reductions of at least 4 log units after 600 MPa for 6 min in sliced vacuum-packaged marinated beef loin, without recovery of survivors during subsequent storage (Garriga et al., 2004). Counts higher than 6 log cfu/g in marinated beef loin were attributed by these authors to inappropriate slaughterhouse operations, handling or chilled storage before processing, reaching 8 log cfu/g after 30 days at 4 °C, whereas in pressurized samples counts were <2 log cfu/g. A recovery of sublethally injured TVC was observed in the present work, but levels in pressurized carpaccio were significantly lower than those in non-pressurized samples during the 30 days of refrigerated storage.

The application of 450 MPa for 5, 10 and 15 min was very effective in inactivating *S.* Enteritidis and reducing TVC, increasing the microbiological safety and extending the shelf-life of carpaccio, even under mild temperature abuse storage conditions.

Table 2
Levels (log cfu/g) of total viable counts (TVC) in pressurized beef carpaccio stored during 30 days at 8 °C.

Time	Non-pressurized	450 MPa /5 min	450 MPa /10 min	450 MPa /15 min
0 h	6.10 ± 0.79^{bA}	3.56 ± 0.38^{abA}	2.98 ± 2.04^{aA}	2.67 ± 1.79^{aA}
15 days	7.10 ± 0.47^{dB}	5.70 ± 1.17^{bB}	6.55 ± 0.37^{cB}	4.65 ± 0.10^{aA}
30 days	7.06 ± 0.23^{dB}	5.73 ± 0.14^{bB}	6.38 ± 0.02^{cB}	4.39 ± 0.13^{aA}

Values are means ± SD.
[a,b,c,d]Means within the same row with different superscripts differ significantly at $P<0.05$.
[A,B,C,D]Means within the same column with different superscripts differ significantly at $P<0.05$.

3.2. pH and a_w

Values of pH and a_w in beef carpaccio stored at 8 °C for 30 days are shown in Table 3. Values of pH were significantly ($P<0.001$) affected by high pressure treatment and storage time.

After pressurization, pH values were between 5.67 and 5.72, without differences ($P<0.05$) between pressurized and control beef carpaccio samples. During refrigeration, pH values decreased in non-pressurized carpaccio whereas changes in pressurized samples were minimal. This fact could be related to the increase of TVC (Table 2) in non-pressurized carpaccio, probably lactic acid bacteria. This microbial group represented 86% of the bacterial population in beef carpaccio, mainly dominated by *Lactobacillus* and *Leuconostoc* (Lucquin, Zagorec, Champomiere-Vergès, & Chaillou, 2011). These authors compared lactic acid bacteria between vacuum-packaged and modified atmosphere-stored beef carpaccio samples, and reported a high prevalence of *Lactobacillus* over *Leuconostoc* in vacuum-packaged samples, showing a well-stabilised population after 14 days at 4 °C with little evolution at longer storage times. These results seem to be consistent with the present findings (Table 3), with a decrease of pH during the first 15 days of storage in non-pressurized carpaccio that was similar to the pH found after 30 days at 8 °C.

Values of a_w increased significantly due to pressurization ($P<0.001$) and time of storage ($P<0.05$) (Table 3). Although higher values ($P<0.05$) of a_w were recorded in treated carpaccio from 15 days under refrigeration at 8 °C, the maximum difference between non-treated and treated carpaccio was 0.01 units, without practical significance in terms of product quality or safety.

High pressure treatment alters the interactions between muscle proteins and water, which subsequently changes the water characteristics of the meat (Bertram, Whittaker, Shorthose, Andersen, & Karlsson, 2004). Other authors have also reported reduced water binding properties in pressurized meat and meat batters (Crehan, Troy, & Buckley, 2000; Jung, Ghoul, & de Lamballerie-Anton, 2000; Mandava, Fernandez, & Juillerat, 1994). Jung, de Lamballerie-Anton, and Ghoul (2000a) related the loss of water binding capacity to more severe shrinkage and myofibrillar changes at higher pressure levels. Sarcoplasmic proteins also have an impact on meat quality and in water binding properties in fresh meat (Joo, Kauffman, Kim, & Park, 1999; Monin & Laborde, 1985). According to Marcos, Kerry, and Mullen (2010), pressure induces denaturation of sarcoplasmic proteins and reduces water holding capacity. McArdle, Marcos, Kerry, and Mullen (2011) reported that HP treatments at 600 MPa diminished the water holding capacity of beef meat.

3.3. Color

Changes in instrumental color characteristics (L^* a^* and b^*) of beef carpaccio are shown in Table 4. High-pressure treatment and time of

Table 3
Values of pH and a_w in pressurized beef carpaccio stored during 30 days at 8 °C.

	Time (day)	Non-pressurized	450 MPa/5 min	450 MPa/10 min	450 MPa/15 min
pH	1	5.68 ± 0.08^{aB}	5.71 ± 0.07^{aA}	5.67 ± 0.04^{aA}	5.72 ± 0.01^{aA}
	15	4.87 ± 0.04^{aA}	5.74 ± 0.07^{bA}	5.70 ± 0.02^{bA}	5.80 ± 0.07^{bA}
	30	4.96 ± 0.15^{aA}	5.72 ± 0.38^{bA}	5.97 ± 0.13^{bB}	5.80 ± 0.12^{bA}
a_w	1	0.965 ± 0.002^{aA}	0.967 ± 0.000^{aA}	0.971 ± 0.002^{aA}	0.969 ± 0.003^{aA}
	15	0.966 ± 0.002^{aA}	0.972 ± 0.001^{bB}	0.976 ± 0.000^{cA}	0.973 ± 0.000^{bcA}
	30	0.965 ± 0.000^{aA}	0.968 ± 0.000^{abA}	0.972 ± 0.001^{bA}	0.973 ± 0.003^{bA}

Values are means ± SD.
[a,b,c,d]Means within the same row with different superscripts differ significantly at $P<0.05$.
[A,B,C,D]Means within the same column with different superscripts differ significantly at $P<0.05$.

Table 4
Color parameters in pressurized beef carpaccio stored during 30 days at 8 °C.

	Time (day)	Non-pressurized	450 MPa/5 min	450 MPa/10 min	450 MPa/15 min
L^*	1	23.65 ± 3.62^{aA}	31.26 ± 2.82^{bA}	33.87 ± 3.00^{bA}	33.71 ± 2.07^{bA}
	15	25.29 ± 1.64^{aA}	33.52 ± 2.19^{bB}	35.95 ± 3.17^{cA}	36.21 ± 2.41^{cB}
	30	25.58 ± 1.66^{aA}	31.17 ± 2.06^{bA}	35.08 ± 2.53^{cA}	36.19 ± 1.76^{cB}
a^*	1	33.47 ± 4.66^{cC}	18.05 ± 1.79^{bB}	14.43 ± 2.63^{aAB}	15.59 ± 1.65^{abB}
	15	21.00 ± 2.88^{cB}	18.40 ± 1.95^{bB}	14.90 ± 2.26^{aB}	14.77 ± 2.03^{aB}
	30	17.58 ± 2.12^{dA}	14.63 ± 1.16^{cA}	12.43 ± 2.01^{bA}	10.23 ± 1.77^{aA}
b^*	1	26.53 ± 3.97^{bB}	15.03 ± 1.96^{aB}	13.50 ± 1.36^{aA}	12.95 ± 0.97^{aB}
	15	14.01 ± 1.78^{bA}	14.28 ± 1.21^{bB}	13.07 ± 1.24^{abA}	12.31 ± 0.96^{aAB}
	30	11.90 ± 1.31^{aA}	12.55 ± 1.00^{aA}	12.34 ± 1.40^{aA}	11.49 ± 1.07^{aA}

Values are means ± SD.
[a,b,c,d]Means within the same row with different superscripts differ significantly at $P<0.05$.
[A,B,C,D]Means within the same column with different superscripts differ significantly at $P<0.05$.

storage at 8 °C affected significantly ($P<0.001$) the values of these parameters.

After HP processing, L^* (lightness) values were higher ($P<0.05$) in pressurized carpaccio than in non-treated samples. This effect was observed throughout the refrigeration period investigated, with a tendency for L^* values to increase in carpaccio pressurized for 15 min. Previous investigations have reported increases in L^* values of beef meat when high pressure treatments were applied at temperatures above 0 °C (Carlez et al., 1995; Jung, Ghoul, & de Lamballerie-Anton, 2003; Marcos et al., 2010) and in fresh or cured minced pork (Goutefongea, Rampon, Nicolas, & Dumont, 1995). These color changes were attributed to globin denaturation and/or heme group displacement or release by Carlez et al. (1995). The structure and surface properties of pressure treated meat would be affected and increase the ratio of reflected versus absorbed light. This increase could be related to myofibrillar and sarcoplasmic protein coagulation (Goutefongea et al., 1995).

Values of a^* (redness) decreased ($P<0.05$) in treated carpaccio with respect to non-treated samples 1 day after treatments. The lowest values ($P<0.05$) were achieved at the end of refrigerated storage in samples pressurized at 450 MPa for 15 min. The decrease in a^* values in pressurized meat products has been related to some changes in the myoglobin molecule such as oxidation of the ferrous myoglobin to ferric myoglobin above 400 MPa (Carlez et al., 1995). In beef carpaccio treated at 600 MPa for 10 min, Franceschini, Gola, Rovere, and Frustoli (2005) also observed a decrease in a^* values. However, this decrease seems not to be as severe in cured meat products as in fresh meat because of the addition of nitrates/nitrites. Pressurization of raw cured meats has shown discoloration problems, with an increase in lightness but minor effects on redness due to the protective action of nitric oxide on myoglobin. Other studies have reported pressure-induced color changes with increased lightness and reduced redness when applied to nitrite-free Parma ham (Tanzi et al., 2004).

After pressurization, b^* (yellowness) values decreased ($P<0.05$) with respect to non-pressurized carpaccio, although these differences were attenuated during storage. Also, b^* values tended to decrease throughout the storage in HP treated and non-pressurized carpaccio samples. Variations of yellowness in meat products have been related to changes in the chemical state of myoglobin (Goutefongea et al., 1995). These authors observed an increase of b^* parameter induced by high pressure in fresh minced beef and pork treated at 600 MPa for 30 min.

Although color of meat is affected by HP treatments as a result of globin denaturation and/or to heme displacement or release and oxidation of ferrous myoglobin to ferric metmyoglobin (Carlez et al., 1995), nitrite was found to prevent such pressure-induced discoloration of minced meat (Goutefongea et al., 1995). In the same way, Bruun-Jensen and Skibsted (1996) found in nitrosylmyoglobin solutions that the rate of oxidation of MbFe(II)NO to MbFe(III) decreased with increasing hydrostatic pressure in the range of 50–350 MPa, indicating a protective effect of high pressure against MbFe(II)NO oxidation. MbFe(II)NO is believed to be an important antioxidant in cured meats due its fast radical exchange reactions (Andersen, Johansen, Shek, & Skibsted, 1990). The discoloration of pressurized beef carpaccio observed in the present work, less severe than that reported in raw meat, could be attributed to the protective action of nitric oxide on myoglobin. On the other hand, Realini et al. (2011) found that pressurization at freezing temperature (−35 °C) may prevent the increase in color lightness without affecting the redness of cured pork carpaccio, although yellowness (b^*) was affected by the treatment. Apparently, freezing protects beef meat against the detrimental effect of pressure on color as the fresh beef meat normal color is recovered after thawing. The mechanism responsible for this protective effect could be explained in terms of high pressure-induced myoglobin denaturation. In pressurized fresh beef meat, myoglobin denaturation would be intense and consequently irreversible. Instead, in frozen beef pressurized at subzero temperature, the effect was probably milder and reversible. Thus, on thawing, myoglobin might recover its native conformation, and consequently, fresh meat normal color could be recovered (Fernández et al., 2007).

3.4. Texture

Texture parameters of pressurized and control beef carpaccio are given in Table 5. According to the analysis of variance, the effect of high-pressure treatment and time of storage on shear strength (N) determined with the Warner–Bratzler blade were statistically significant ($P<0.001$). After treatment, shear strength (N) decreased ($P<0.05$) in pressurized carpaccio compared to control samples, although differences were lower when the time of pressurization increased. Shear strength (N) values increased significantly ($P<0.05$) in treated samples during storage.

Maximum force (N) was significantly affected by treatments ($P<0.05$) and time of storage ($P<0.001$). After treatment, no differences were detected between non-pressurized and HP treated carpaccio. Hardness of beef carpaccio determined with the Kramer cell also

Table 5
Texture properties of pressurized beef carpaccio stored during 30 days at 8 °C.

	Time (day)	Non-pressurized	450 MPa/5 min	450 MPa/10 min	450 MPa/15 min
Shear force (N)	1	46.2 ± 14.1^{cA}	21.0 ± 5.6^{aA}	29.1 ± 4.1^{abA}	35.5 ± 9.3^{bcA}
	15	32.3 ± 10.1^{aA}	35.9 ± 8.5^{aB}	44.6 ± 21.6^{aB}	43.0 ± 6.5^{aA}
	30	40.8 ± 18.7^{aA}	37.1 ± 12.7^{aB}	66.3 ± 8.5^{bC}	65.8 ± 11.6^{bB}
Maximum force (N)	1	66.7 ± 32.0^{aA}	62.0 ± 10.4^{aA}	71.6 ± 23.3^{aA}	75.7 ± 21.5^{aA}
	15	91.5 ± 29.7^{aA}	97.8 ± 49.2^{aA}	77.1 ± 23.6^{aA}	85.3 ± 28.5^{aA}
	30	79.1 ± 16.9^{aA}	154.8 ± 37.0^{cB}	134.1 ± 38.6^{bcB}	96.6 ± 41.3^{abA}

Values are means ± SD.
[a,b,c,d]Means within the same row with different superscripts differ significantly at $P<0.05$.
[A,B,C,D]Means within the same column with different superscripts differ significantly at $P<0.05$.

increased during storage, mainly in the treated product, and the highest values were observed at 450 MPa for 5 and 10 min after 30 days at 8 °C.

Depending upon the meat protein system, the pressure, the temperature, and the duration of the high pressure treatment, meat can be either tenderized or toughened by HP. Suzuki, Kim, Homma, Ikeuchi, and Saito (1992) reported a decrease in the resistance force of post-rigor meat after pressurization at 100, 150 and 300 MPa for 5 min at 10 °C. Changes in contractile myofibrillar proteins are thought to be primarily responsible for differences in the textural properties of HP treated meat (Ma & Ledward, 2004), with ultrastructural changes in myofibrils noticed at pressures above 325 MPa (Jung, de Lamballerie-Anton, & Ghoul, 2000b). High pressure could affect the integrity of lysosomes (Jung, de Lamballerie-Anton, Taylor, & Ghoul, 2000) and increase cathepsin D and acid phosphatase activities in pressurized beef, influencing its textural characteristics (Jung et al., 2000).

Tenderness seems to be the most important factor of all the attributes that characterize the eating quality of meat (Denoyelle & Lebihan, 2004; Jung et al., 2000a). Further studies on sensory analysis should be carried out to determine the consumer acceptability of pressurized carpaccio under the conditions investigated in the present work.

4. Conclusions

High-pressure treatments at 450 MPa for 5, 10 and 15 min, were very effective in inactivating *Salmonella* Enteritidis inoculated on beef carpaccio. Antimicrobial effects remained over 30 days under temperature abuse conditions (8 °C). A slight whitening of carpaccio and lower redness was observed in pressurized samples, although this effect was higher when the time of pressurization increased. Also, hardness (N) increased at the end of the refrigerated period with the more intense treatments. Taking into account the high *Salmonella* load inoculated on beef carpaccio in the present work and the temperature abuse conditions studied, treatments at 450 MPa for 5 min, that reduce *Salmonella* counts by 5 log units might be applied by the meat industry to extend the shelf-life of beef carpaccio. Further studies on combined treatments to improve beef carpaccio safety, reducing pressurization intensities to minimize organoleptic changes will be carried out.

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3.5. Combined treatments of high-pressure with the lactoperoxidase system or lactoferrin on the inactivation of *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* O157:H7 in beef carpaccio. *Food Microbiology* (Aceptado)

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ABSTRACT

The effect of high hydrostatic pressure (HHP) treatments in combination with the lactoperoxidase system (LPOS) or activated lactoferrin (ALF) on *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Escherichia coli* O157:H7 was investigated in cured beef carpaccio stored at 8 °C or 22 °C during 7 d. HHP (450 MPa for 5 min) reduced pathogen levels by 1–3 log units and the antimicrobial effect remained during 7 d of storage under temperature abuse conditions, at 8 °C and 22 °C. The individual application of LPOS and ALF did not affect the survival of the three pathogens studied during storage. However, a synergistic bactericidal interaction between LPOS and HHP was observed against *S.* Enteritidis and *E. coli* O157:H7. Combined treatments of HHP with LPOS would be useful to reduce the intensity of pressurization treatments diminishing changes in the quality of meat products.

1. Introduction

Beef carpaccio is a ready to eat product (RTE) that can be contaminated with foodborne pathogens during its processing. Raw or cured pieces of meat usually frozen are sliced, packaged under vacuum or modified atmospheres and marketed at refrigeration temperature. The risk of the product increases when stored at temperature abuse conditions. *Listeria monocytogenes* can persist in the processing environment adhered to stainless steel surfaces which act as reservoir of contamination. *Salmonella* is found in the environment and in the gastrointestinal tract of farmed and wild animals. *Escherichia coli* O157:H7, a verocytotoxin producing *E. coli* (VTEC), associated with both outbreaks and sporadic cases, can be shed in animal faeces and contaminate the surfaces of raw meat during the slaughter, dressing and packaging. The incidence of reported foodborne illness for *L. monocytogenes* and VTEC decreased, whereas for salmonellosis increased since 2006–2008 in the United States (CDC, 2011). In the European Union, cases attributed to *L. monocytogenes* and *Salmonella* have decreased during the last years, but VTEC infections have increased since 2008 (EFSA and ECDC, 2013). RTE meat products stored at temperature abused conditions can result in spoiled or unsafe food relatively early due to faster multiplication of microorganisms. According to Pal et al. (2008) in the event of contamination of deli meat and poultry food with *L. monocytogenes* even with a lowest detectable level, the pathogen was able to grow especially at 8 and 12 °C.

High hydrostatic pressure (HHP) is a non-thermal technology applied to increase the microbial safety and prolong the shelf-life of foods. In RTE meat products, HHP as a post-processing treatment after packaging provides an additional barrier to assure microbiological safety (Rendueles et al., 2011), and also is considered a novel strategy to alter the structure and quality parameters of meat and meat products (Bajovic et al., 2012). Reductions of *S.* Enteritidis of 3.7–5.9 log units in beef carpaccio pressurized at 450 MPa for 5 and 10 min, respectively, were reported by De Alba et al. (2012), although changes in colour were detected at longer pressurization times. In general, HHP induced colour changes are more intense in fresh red meat than in white meat and cured meat products (Bajovic et al., 2012), that in carpaccio could be attributed to the protective action of nitrite (Szerman et al., 2011; De Alba et al., 2012).

High pressures combined with lysozyme or with some bacteriocins exhibited a synergistic antimicrobial effect against pathogens (Hauben et al., 1996), and were considered a useful strategy to

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reduce intensity of pressurization treatments. The combination of pressurization with other biopreservatives as the lactoperoxidase system (LPOS) or lactoferrin in meat products has not been thoroughly studied. Lactoperoxidase catalyses the oxidation of thiocyanate (SCN^-) by hydrogen peroxide (H_2O_2) to hypothiocyanite ($OSCN^-$), an unstable antimicrobial compound that oxidize exposed sulfhydryl group of enzymes and proteins in the bacterial cell membrane, interfering the transport of nutrients, the DNA and RNA synthesis and the respiratory chain (Pruitt and Reiter, 1985). LPOS has been approved as processing aid for meat and meat products by the Food Standards Australia New Zealand (FSANZ, 2002) and proposed by FAO/WHO (2005) for preventing the microbial growth in milk. Lactoferrin is a single-chain iron-binding glycoprotein that constitutes one of the major antimicrobial systems in milk. Antimicrobial activity is due to the iron-binding properties of the protein. Activated lactoferrin (ALF) is an immobilized lactoferrin patented by Naidu (2001).

The aim of this study was to evaluate the effect of HHP combined with the LPOS or ALF on the survival of inoculated *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 on cured beef carpaccio stored under temperature abuse conditions at 8 °C and 22 °C during 7 days.

2. Materials and methods

2.1. Microorganisms

L. monocytogenes INIA H66a (from the INIA Culture Collection, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain), *Salmonella enterica* subsp. *enterica* serovar Enteritidis strains CECT 4155, CECT 4300, and CECT 4396, and *E. coli* O157:H7 CECT 4972 (from the Spanish Type Culture Collection, Valencia, Spain) were used. The strains were kept frozen at −80 °C in trypticase soy yeast extract broth (TSYEB) (Biolife, Milano, Italy) with 30% glycerol. Samples were inoculated with an over-night culture grown in TSYEB at 37 °C for 18 h.

2.2. Preparation of samples

Cured beef carpaccio was purchased from a retail market in Madrid (Spain) and kept frozen until used. Slices were aseptically cut into 10 g pieces 2 mm thick and inoculated by spreading 100 μL of diluted over-night cultures of *L. monocytogenes*, *S.* Enteritidis or *E. coli* O157:H7. Inoculated samples were individually vacuum-packed in double bags of BB325 (Cryovac Sealed Air Corporation, Milan, Italy) and held at 4 °C for 18 h until applying the individual or combined treatments.

2.3. HHP processing

High-pressure treatments were performed in a prototype ACIP 6000 (ACB, Nantes, France) of 3.5 L capacity and 600 MPa maximum working pressure. Water was used as pressure transmitting medium. Samples were pressurized at 450 MPa for 5 min at 12 °C. All samples were stored under refrigeration at 8 °C and 22 °C during 7 days. Two independent trials were carried out.

2.4. Application of lactoperoxidase system and activated lactoferrin

Lactoperoxidase from bovine milk (DMV International, Veghel, Holland) was prepared in distilled-deionized water (15 mg/ml) sterilized with 0.22 μm pore size cellulose acetate filters (Millipore, Bedford, MA) and stored at −40 °C. The oxidation of ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (Sigma–Aldrich, Alcobendas, Spain) was used to measure lactoperoxidase activity at 412 nm that was expressed in ABTSU (Shindler et al., 1976). The lactoperoxidase system was activated by adding 2.8 ABTSU/g, 0.03 mg/g potassium thiocyanate (KSCN, Merck, Darmstadt, Germany), 0.16 mg/g glucose and 0.15 U/g glucose oxidase. Activated lactoferrin (ALF) obtained from DMV International was prepared at 20 mg/ml in double distilled water, sterilized with 0.22 μm pore size cellulose acetate filters and stored at −40 °C until used. Inoculated carpaccio samples were treated with 1 mg/g. Antimicrobials were applied by spreading on the carpaccio surface.

2.5. Microbiological analysis

Samples (10 g) were transferred aseptically to a sterile stomacher bags, diluted 10-fold with sterile 0.1% (wt/vol) peptone water solution and homogenized for 90 s in a stomacher 400 (A. J. Seward Ltd., London, UK). Decimal dilutions were prepared in the same solution and spread on duplicate plates. Chromagar Listeria (CHROMagar, Paris, France) for *Listeria*, Salmonella Shigella agar (SSA, Scharlab S.L., Barcelona, Spain) for *Salmonella* and Violet Red Bile agar (VRBA, Oxoid LTD., Basingstoke, Hampshire, England) for *E. coli* O157:H7 were incubated at 37 °C for 24 h.

2.6. Statistical analysis

Data were subjected to analysis of variance (ANOVA) of SPSS program Win 12.0 software (SPSS Inc., Chicago, IL). Significant differences between means were assessed by the Tukey's test ($P < 0.05$).

3. Results

3.1. Effect of treatments on *L. monocytogenes* population

Survival of *L. monocytogenes* in cured beef carpaccio subjected to HHP at 450 MPa for 5 min, LPOS or ALF and their combinations, and stored at 8 or 22 °C is shown in Fig. 1. Immediately after treatments, pressurization led to mean reductions of 1.1 log cfu/g, whereas no antimicrobial activity was observed when LPOS or ALF were added to carpaccio. Reductions of *L. monocytogenes* achieved by combined treatments of HHP with LPOS or ALF were 1.4 and 1.0 log units, respectively. During the refrigerated storage, *L. monocytogenes* did not grow in control carpaccio during 7 d at 8 °C, with a decrease of 0.2 log cfu/g. Reductions of 1.7 log cfu/g were obtained in pressurized carpaccio, which attained 2.4 and 1.9 log units when HHP and LPOS or ALF were combined, respectively. After 7 d at 22 °C, reduction in counts of *L. monocytogenes* was 2.4 log cfu/g in samples treated by HHP. Counts in carpaccio pressurized in presence of LPOS or ALF were 2.9 and 1.8 log units lower than in control carpaccio. However, no significant differences in *L. monocytogenes* levels were detected between non-pressurized, LPOS or ALF treated carpaccio throughout storage at 8 or 22 °C.

3.2. Effect of treatments on *S. Enteritidis* population

S. Enteritidis counts in cured beef carpaccio subjected to HHP at 450 MPa, LPOS, ALF or their combinations and stored at 8 or 22 °C are shown in Fig. 2. After treatment, the reduction in the pathogen counts was 2.7 log cfu/g in carpaccio pressurized at 450 MPa for 5 min. Differences in *S.* Enteritidis counts in non-pressurized carpaccio and in samples treated with LPOS or ALF were not significant. When HHP was combined with LPOS or ALF, the inactivation observed for *S.* Enteritidis was 3.6 and 2.7 log units. At the end of the 7 d of refrigeration at 8 °C, counts of *S.* Enteritidis decreased by 0.9 log cfu/g in non-pressurized samples and 3.7 log cfu/g in pressurized samples. The inactivation was higher

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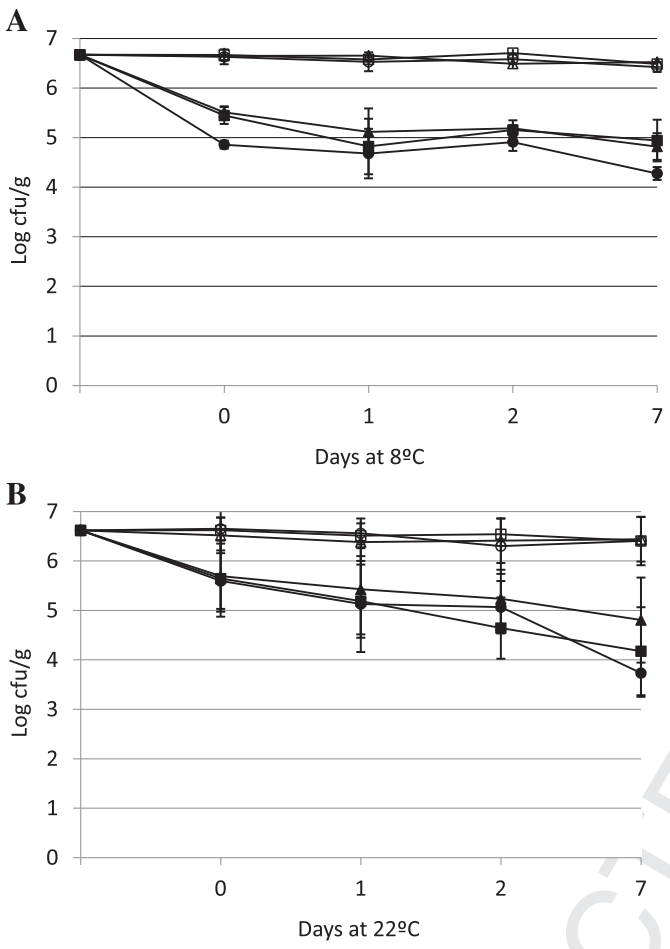

Fig. 1. *Listeria monocytogenes* counts in carpaccio at 8 °C (A) or 22 °C (B) during 7 days. Control (□), LPOS (○), ALF (△), HHP at 450 MPa (■), HHP + LPOS (●), and HHP + ALF (▲).

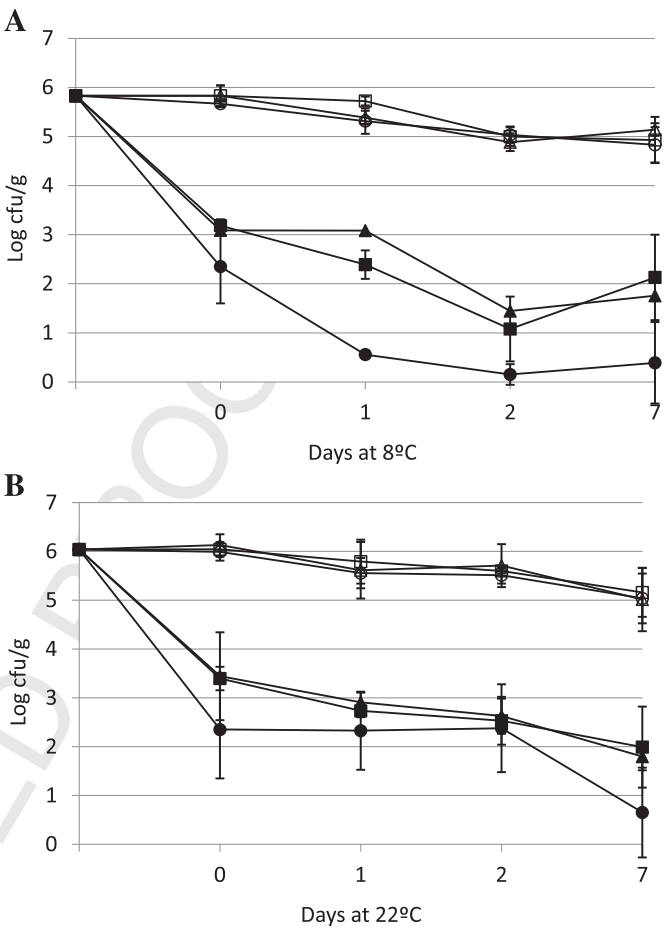

Fig. 2. *Salmonella* Enteritidis counts in carpaccio at 8 °C (A) or 22 °C (B) during 7 days. Control (□), LPOS (○), ALF (△), HHP at 450 MPa (■), HHP + LPOS (●), and HHP + ALF (▲).

after applying HHP combined with LPOS or ALF, achieving reductions of 5.4 and 4.1 log units, respectively. After 7 d at 22 °C, counts of *S.* Enteritidis decreased by 0.9 log units in control cured beef carpaccio samples. In pressurized samples counts decreased by 4.1 log units, and reductions of 5.4 and 4.2 log cfu/g were detected when HHP plus LPOS or ALF were applied. A slight synergistic antimicrobial effect against *S.* Enteritidis was observed in pressurized samples combined with LPOS stored at both 8 and 22 °C, being stronger at 8 °C.

3.3. Effect of treatments on *E. coli* O157:H7 population

Counts of *E. coli* O157:H7 in cured beef carpaccio subjected to individual or combined treatments and stored at 8 or 22 °C are shown in Fig. 3. The reduction in the levels of *E. coli* O157:H7 immediately after HHP treatment was 1.6 log cfu/g. No significant differences in counts were observed in control, LPOS or ALF treated samples. When HHP was combined with LPOS or ALF, the reduction attained was 2.0 and 1.7 log cfu/g, respectively. Counts decreased at day 1 but after this short term inhibitory effect *E. coli* cells tend to recover at both temperatures. At the end of the refrigeration at 8 °C, counts of *E. coli* O157:H7 did not decrease in non-pressurized samples and diminished 2.1 log cfu/g in HHP treated carpaccio. The combination of HHP with LPOS or ALF led to counts 2.1 and 2.4 log cfu/g lower than in control carpaccio, respectively. After 7 d at 22 °C, *E. coli* O157:H7 counts were not reduced in non-pressurized samples. However, counts in samples pressurized at 450 MPa for 5 min were 2.8 log cfu/g lower than in control carpaccio. Combined treatments with LPOS or ALF led to 4.7 and 3.0 log cfu/g reductions, respectively. A strong synergistic antimicrobial activity against *E. coli* O157:H7 was evidenced in pressurized samples combined with LPOS and stored at 22 °C after 7 d. This effect was not detected at 8 °C, with similar levels of the pathogen in samples pressurized and after pressurization and LPOS application. According to our results, the ability of HHP plus LPOS to inhibit the growth of *E. coli* O157:H7 could be temperature dependent. A recovery of growth of injured cells was detected in both experiments at 8 and 22 °C, although the effect was more apparent at the lower temperature.

4. Discussion

Contamination of RTE meat products with pathogenic microorganisms during processing, storage and distribution represents a risk for consumers and a major concern for the food industry. Different strategies for preventing the growth of contaminant pathogens in foods will continue being needed. The effect of HHP on foodborne pathogens and factors affecting microbial resistance has been reviewed by Rendueles et al. (2011). The implementation of the technology in the meat processing industry provides safer, higher quality, and higher value products. HHP technologies in combination with biopreservatives have been proposed to enhance

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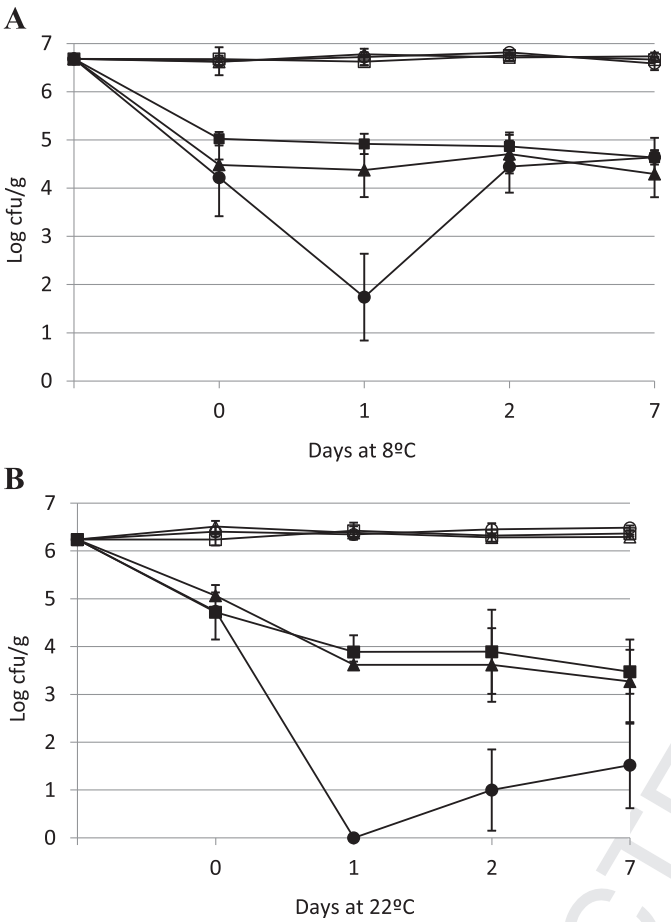

Fig. 3. *Escherichia coli* O157:H7 counts in carpaccio at 8 °C (A) or 22 °C (B) during 7 days. Control (□), LPOS (○), ALF (△), HHP at 450 MPa (■), HHP + LPOS (●), and HHP + ALF (▲).

bacterial inactivation in the hurdle approach for food safety (Leistner, 2000). However, depending on the pressure intensity and the product, pressurization causes whitening effects and important decreases in redness. The lactoperoxidase system and lactoferrin are natural antimicrobial agents present in milk with a broad spectrum of antimicrobial activity against different microorganisms potentially present in foods (Naidu, 2000).

In the present work, growth of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 was not detected in inoculated carpaccio throughout the 7 days of storage at 8 or 22 °C studied. Antimicrobial activity of the curing mixture present in this product would restrict the growth of pathogens even at 22 °C.

HHP exhibited a bactericidal effect, with decreases between 1.1 and 2.7 log units, depending on the pathogen, whereas LPOS or ALF were not active when applied individually. HHP processing at 450 MPa for 5 min showed the higher sensitivity of *S.* Enteritidis to the treatment, followed by *E. coli* and *L. monocytogenes*, which exhibited higher barotolerance in carpaccio. Gram-negative bacteria are considered more sensitive to high pressure treatments than Gram-positives. Different strains of *Salmonella* inoculated on cooked ham were more sensitive to HHP treatments than *E. coli* (Garriga et al., 2002). Also, *E. coli* O157:H7 has been characterized as a pressure resistant serotype (Benito et al., 1999). Reductions of *S.* Enteritidis up to 5 log units in beef carpaccio pressurized at 450 MPa for 5 or 10 min were reported by De Alba et al. (2012), although changes in colour were detected at the longer pressurization time. Bactericidal activity of HHP treatments was higher in chicken meat than in carpaccio, with decreases of 4.8 log units for *L. monocytogenes* after 400 MPa for 10 min (Del Olmo et al., 2012a) and more than 5 log cfu/g for *E. coli* O157:H7 (Del Olmo et al., 2012b). Inactivation of pathogens by HHP depends on a number of factors including the Gram type, the physiological state and the strain assayed, the product characteristics as pH, salt content, water activity, the presence of other ingredients, and the processing conditions. According to Bajovic et al. (2012), inactivation of more than 4 log units for the most common vegetative pathogens can be attained in cooked meat products and fresh meat at 400–600 MPa for 3–7 min at room temperature, although unwanted changes at pressures above 400 MPa can be observed. The HHP antimicrobial effect can be lower in cured meat products due to the higher resistance of pathogens to this treatment at low a_w values (Hereu et al., 2012).

Bacteriostatic or bactericidal activities for LPOS in foods have been reported. The antimicrobial effect is due to the oxidation of sulfhydryl groups of bacterial enzymes and other proteins in bacterial membranes. The LPOS activated in refrigerated milk was bactericidal against *L. monocytogenes* (Zapico et al., 1998; Arqués et al., 2008), although according to García-Graells et al. (2000) bactericidal activity in milk was only detected on *Pseudomonas fluorescens*, whereas no effect or a growth inhibiting activity occurred against other foodborne bacteria. Fewer studies on the application of LPOS in meat products have been published. When the LPOS was added at 1.9 U/cm^2 to fresh meat, the antimicrobial activity was bacteriostatic, preventing the growth of pathogenic bacteria at 12 °C and reducing it at chilling temperatures (Elliot et al., 2004). In ground beef, although different pathogens were able to grow at refrigeration temperatures, large variations on the antimicrobial activity were observed, depending on the strain, the inoculation rate and the temperature, with maximal inhibition at temperatures permissive but not optimal for growth (Kennedy et al., 2000). LPOS addition to smoked salmon reduced *L. monocytogenes* counts by one log unit, and the pathogen reinitiated its growth from day 2 of LPOS activation in samples at 5 °C (Montiel et al., 2012). In the present work, the LPOS was not active against pathogens inoculated in carpaccio. The lower effectivity of the LPOS system in egg or chicken meat compared with vegetable juices was attributed to the binding of the LPOS to fats and proteins or to the absorption of $OSCN^-$ to food components (Touch et al., 2004). Also, when medium or high populations of *L. monocytogenes* were present in the sample, the LPOS failed to cause pathogen inhibition (El-Shenawy et al., 1990).

Antimicrobial activity of lactoferrin and its derivatives is limited in complex food systems. Slight bactericidal effect of ALF has been reported against *L. monocytogenes* in chicken filets (Del Olmo et al., 2012a) and against *E. coli* O157:H7 inoculated in chicken meat (Del Olmo et al., 2012b). Bactericidal activity of lactoferrin and derivatives that was high when assayed in water practically disappeared when added at 1 mg/g to ground beef inoculated with *P. fluorescens*. The loss of activity observed in this product, in ground beef homogenates and in dialysis water was attributed to compounds of low molecular mass, as in the dialysate the antimicrobial activity was restored. The presence of low molecular compounds like cations in meat samples, might decrease or even abolish the antimicrobial activity of lactoferrin and derivatives (Del Olmo et al., 2009).

In the present work, antimicrobial activity of LPOS or lactoferrin applied individually on the surface of carpaccio was not detected, fact that might be related to an irregular spreading of the antimicrobials, the interference with meat components or curing agents present in carpaccio, or the inoculum size, as previously reported by Kennedy et al. (2000) in ground beef treated with the LPOS or by Al-Nabulsi and Holley (2007) for lactoferrin in dry sausage batters.

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The concentration of hypothiocyanite per target cell could limit the activity of the LPOS, and the presence of cations the antimicrobial effect of lactoferrin.

The cooperative antimicrobial activity of HHP and the LPOS on *L. monocytogenes* inactivation in milk was demonstrated by García-Graells et al. (2000), and confirmed on several foodborne bacteria (García-Graells et al., 2003). According to these authors, pressure and LPOS resistance observed for the baroresistant *E. coli* LMM1010 might be associated to oxidative stress resistance, as the oxidation of sulfhydryl group of enzymes and proteins in the bacterial cell membrane is the main activity of LPOS. In this sense, Aertsen et al. (2005) observed that HHP treatments make *E. coli* cells hypersensitive to subsequent oxidative stress. High-pressure homogenization at 300 MPa did not sensitize *E. coli* cells in buffer to the action of the LPOS. According to Diels et al. (2005), the hydrostatic pressure component was sufficient to cause transient outer membrane permeabilisation, but not to cause sensitivity to oxidative stress or any metabolic injury, which would need higher pressures. On the contrary, transient permeabilisation of the outer membrane by high-pressure homogenization sensitized *E. coli* to lysozyme and nisin. Other authors (Vannini et al., 2004) reported a synergistic effect of high pressure homogenization up to 130 MPa and the LPOS in milk against *E. coli* and *L. monocytogenes* at 37 °C, that was associated to conformational modifications of the antimicrobial enzyme with a subsequent enhancement of its activity. In the present work, a synergistic antimicrobial effect between HHP at 450 MPa for 5 min and LPOS was observed against *S.* Enteritidis and *E. coli* O157:H7 in carpaccio just after applying treatments and at 7 d when beef carpaccio samples were kept at 8 and 22 °C. As the antimicrobial activity of the LPOS relies on the impairment of metabolic activities, its effect may be reduced at the lower temperature in which bacteria are not metabolically active. Lower reductions of *S.* Enteritidis were observed in liquid whole egg and chicken skin extracts at low (15 °C) than at high (30–37 °C) temperatures, related by Touch et al. (2004) to changes in the fluidity of the bacterial membranes.

The main mechanism of action of lactoferrin is based on electrostatic interactions with lipopolysaccharide molecules altering the outer membrane permeability in Gram negatives, or with teichoic or lipoteichoic acids in gram-positive bacteria (Del Olmo et al., 2012b). The combination with activated lactoferrin slightly reinforced the bactericidal effect of HHP against *E. coli* O157:H7 in chicken meat, with additional reductions of 0.1–0.3 log units to the 5 log cfu/g achieved by treatments at 400 MPa for 10 min (Del Olmo et al., 2012b). No synergistic interaction between HHP and ALF was detected on this pathogen, whereas the antimicrobial effect of the combined treatment was synergistic against *P. fluorescens*. Differences in resistance of gram-positives to lactoferrin and derivatives were detected by Del Olmo et al. (2012a), with maximum sensitivity *in vitro* for *L. monocytogenes* that was below 0.5 log units in chicken meat. In combination with HHP at 400 MPa for 10 min, a slight additional bactericidal activity lower than 0.5 log units was detected. A transient sensitization of gram-negatives to lactoferrin and derivatives by HHP treatments at 400 MPa for 15 min in phosphate buffer was reported by Masschalck et al. (2001). No enhancement of the antimicrobial potential of HHP was detected in the present work when this technology was applied in combination with ALF.

According to our results, high-pressure treatments at 450 MPa during 5 min were effective on the inactivation of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 inoculated on the surface of cured beef carpaccio. The antimicrobial effect remained during 7 d of storage under temperature abuse conditions, at 8 °C and 22 °C. However, no antimicrobial activity was detected by the biopreservatives LPOS or ALF applied individually. A synergistic antimicrobial effect was observed between high hydrostatic pressure treatments and the LPOS application against *Salmonella* Enteritidis and *E. coli* O157:H7. At the end of 7 d of storage, higher reductions were observed at 22 °C after combined treatments of HHP with LPOS for *L. monocytogenes* and *E. coli* O157:H7. However, for *Salmonella*, the effect was more intense at 8 °C.

Considering that pathogen levels hardly could reach the cell density assayed in the present work, combined treatments of HHP and LPOS would contribute to improve the microbiological safety of beef carpaccio. Even though temperature abuse conditions could permit a recovery and proliferation of the pathogens in this product, the LPOS could be more efficient at 22 than 8 °C, avoiding the recovery of damaged cells. Our results confirm that HHP is an attractive non-thermal process to reduce the presence of pathogens in cured beef carpaccio. Although HHP might cause undesirable quality changes in fresh meat, these defects are low in carpaccio and depend on the pressure level, being lower at lower pressurization intensities. The application of HHP combined with LPOS would be useful to reduce the intensity of pressurization treatments with minimal changes in the quality of meat products.

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3.6. High pressure treatments combined with the lactoperoxidase system or activated lactoferrin on cured beef carpaccio quality. *Meat Science* (Manuscrito en preparación).

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High pressure treatments combined with the lactoperoxidase system or activated lactoferrin on cured beef carpaccio quality

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Running title: High pressure and lactoperoxidase system or activated lactoferrin in beef carpaccio.

Abstract

The effect of high hydrostatic pressure (HHP) treatment at 450 MPa for 5 min, the lactoperoxidase system (LP system) or activated lactoferrin (ALF) and their combinations on total viable counts (TVC), pH, a_w, color and texture characteristics of cured beef carpaccio stored during 30 d at 8 ºC was investigated. Immediately after pressurization, reductions higher than 3 log cfu/g were achieved in TVC levels with all treatments. An increase of counts was observed at the end of storage, although TVC (log cfu/g) were at lower levels in treated samples than in non-treated beef carpaccio, achieving the highest reductions with combined treatments. Lightness (L^*) increased with HHP, applied alone or combined with antimicrobials, although these differences were attenuated at the end of storage. Immediately after pressurization, redness (a^*) values hardly were affected by treatments. Yellowness (b^*) decreased with most of treatments and shear strength values increased in samples treated with antimicrobials applied alone at the end of storage. The highest values of hardness were observed with the LP system after 30 d at 8 ºC.

Keywords: Beef carpaccio, high pressure, lactoperoxidase system, activated lactoferrin, texture, color.

Introduction

Consumers prefer fresh and nutritious foods, with a long shelf-life and easy preparation. This trend has increased the interest in minimally-processed products such as ready-to-eat (RTE) over the past years. Cured beef carpaccio is a RTE meat product that is prepared from raw or cured pieces of meat, which are frozen, sliced, packaged under vacuum or modified atmosphere and marketed at refrigeration temperatures. It can be contaminated in processing steps as slicing and packaging. Since carpaccio is consumed without further higienization it could be considered of concern from the safety point of view.

High hydrostatic pressure (HHP) treatment is used as an effective post-packaging decontamination technology for RTE meat products, particularly in cases where heat treatment is not possible or convenient, resulting in an increased shelf-life and improved safety of pressurized products.

The lactoperoxidase system (LP system) is considered as one of the body's natural defence mechanisms against microbial infections. The LP system has a broad antimicrobial spectrum, showing bactericidal and bacteriostatic effect on Gram-negative and Gram-positive bacteria, respectively (Seifu, Buys, & Donkin, 2005). The components of the LP system are the enzyme lactoperoxidase, a source of isothiocyanate ions, either potassium or sodium isothiocyanate and hydrogen peroxide (H_2O_2). The presence of glucose oxidase (GOX) and its substrate glucose allows H_2O_2 required by the LP system to be continuously generated and replenished (Seifu et al., 2005). It has been reported that a more sustained inhibitory effect is obtained if the H_2O_2 is generated *in situ* by added GOX and glucose (Earnshaw, Banks, Defrise, & Francotte, 1989). The enzyme catalyzes the oxidation of thiocyanate (SCN^-) by use of H_2O_2 and generates highly reactive intermediate antimicrobial products. The antimicrobial effect is due to the oxidation of sulphydryl (-SH) groups, enzyme systems and proteins (Kussendrager, & van Hooijdonk, 2000).

The structural damage of microbial cytoplasmic membranes is the principal reason that causes the death of microbial cells (Kussendrager et al., 2000).

Lactoferrin (LF) is a single-chain iron binding glycoprotein of approximately 80 kDa molecular mass found in many secretions of mammals and with antimicrobial activity (Jenssen & Hancock, 2009; Legrand et al., 2008; Naidu, 2000). Activated lactoferrin (ALF), also known as stabilized or immobilized LF, is the result of a patented technology (Naidu, 2001). Its commercial form, Activin™, is formulated for enhancing antimicrobial functions by using a specific molecular-milieu optimization process that includes in its formulation citrate, bicarbonate, EDTA, sodium chloride, immobilized LF polysaccharides via cationic N-terminus, and an excess of unbound LF (Naidu, 2001, 2002).

Although some studies have been conducted on the use of the LP system, LF and its derivatives or HHP, applied alone (Kennedy, O´Rourke, McLay, & Simmonds, 2000; Elliot, McLay, Kennedy, & Simmonds, 2004; Del Olmo, Morales, & Nuñez, 2009; Realini, Guàrdia, Garriga, Pérez-Juan, & Arnau, 2011; Szerman et al., 2011; Vaudagna et al., 2012) or combined (Del Olmo, Calzada, & Nuñez, 2012a,b; Montiel, Bravo, De Alba, Gaya, & Medina, 2012) in meat products and seafood, to our knowledge, the application of this non-thermal technology combined with the LP system or ALF on the quality of cured beef carpaccio, stored at mild temperature abuse condition (8 ºC) (Garrido, García-Jalón, & Vitas, 2010), has not been investigated.

The aim of this work was to evaluate the effect of HHP, the LP system or ALF and their combinations on microbiological, physicochemical, rheological and color characteristics of cured beef carpaccio stored during 30 d at 8 ºC.

2. Materials and methods

2.1. Sample preparation

Beef carpaccio was obtained from a local market in Madrid (Spain). Samples were aseptically prepared to determine total viable counts (TVC), pH, a_w, texture and color. Beef carpaccio was sliced into 2-mm-thick slices to determine texture and color characteristics.

2.2. Preparation and addition of biopreservatives

Lactoperoxidase from bovine milk (DMV International, Veghel, Netherlands) was prepared in distilled-deionized water (15 mg/mL), sterilized with 0.2 μm-pore-size cellulose acetate filters (Millipore, Bedford, MA) and stored at -40 ºC. LP activity was quantified according to the method of Marshall, Cole, and Bramley (1986) by determination of the oxidation of ABTS (2,2´-azino-bis-[3-ethylbenzthiazoline-sulphonic acid]) (Sigma-Aldrich, Alcobendas, Spain) at 412 nm and expressed in ABTS units (Shindler, Childs, & Bardsley, 1976). Potassium thyocianate (KSCN, Merck, Darmstadt, Germany) was prepared in a 1 M aqueous solution and autoclaved at 121 ºC for 15 min. Glucose (Panreac, Barcelona, Spain) stock (1 M) and Glucose oxidase (Sigma-Aldrich) stock at a concentration of 2260 U/mL were prepared according to Kennedy et al. (2000). They were sterilized by means of 0.2 μm-pore-size cellulose acetate filters and stored at room temperature and at 4 ºC until use, respectively. The LP system was activated *in vitro* by adding LP to reach an estimated final activity in carpaccio of 2.8 ABTS U/g followed by 0.03 mg/g of KSCN, 0.16 mg/g of glucose and 0.15 U/g of GOX. Immediately, the activated LP system was added by spreading on the carpaccio surface.

Activated lactoferrin in its commercial form (Activin^{TM}) was obtained from DMV International (Barcelona, Spain). A 20 mg/mL solution of ALF in distilled-deionized water was used as the stock solution for assay, sterilized by means of 0.2 μm-pore-

size cellulose acetate filters and stored at -20 ºC until use. ALF at a concentration of 1 mg/g was added to each sample by spreading on the surface.

2.3. HHP processing

Treated and non-treated samples were individually vacuum-packaged in double bags of BB325 (Oxygen permeability 25 cm^3/m^2; Cryovac Grace S.A., Barcelona, Spain). Samples were pressurized at 450 MPa and one holding time (5 min) at approx. 12 ºC. HHP treatment were performed in a high-pressure prototype ACIP 6000 (ACB, Nantes, France) of 3.5-L capacity and 600 MPa maximum working pressure. Water was used as a pressure transmitting medium.

Experimental treatments were the LP system, ALF, 450 MPa for 5 min, 450 MPa for 5 min combined with the LP system or ALF, and non-treated control group. After pressurization, treated and non-treated samples were stored under refrigeration at 8 ºC during 30 d. Two independent trials were performed on two different days.

2.4. Microbiological analysis

TVC were determined immediately after HHP treatment (time 0) and at 1, 7, 15 and 30 d of storage. Samples (10 g) were homogenized with 90 mL of sterile 0.1% (wt/vol) peptone water solution in stomacher bags for 90 s using a homogenizer (IUL, Barcelona, Spain). Decimal dilutions of the homogenate were prepared in the same sterile solution and TVC were determined on duplicate plates of tryptic soy agar (TSA) (Biolife) incubated at 30 ºC for 72 h.

2.4. pH and a_w

Beef carpaccio samples (5 g) were homogenized with 45 mL of distilled water in stomacher bags for 90 s and pH determinations were taken using a pH-meter (model GPL 22, Crison Instruments, Barcelona, Spain). Water activity (a_w) was measured in carpaccio samples using the AquaLab Series 3 equipment (Decagon Devices, Inc.,

Pullman, WA, EE.UU.). Samples were taken at 0 h, 1, 7, 15 and 30 d after pressurization. Two measurements were carried out per sample.

2.5. Color measurements

Surface color of beef carpaccio was measured in different locations and the respective mean of six measurements per sample was expressed as the final value using a Minolta CM 700d Chromometer (Minolta Camera Co., Osaka, Japan). *L** (lightness), *a** (redness) and *b** (yellowness) were determined at 0 h, 7, 15 and 30 d after pressurization.

2.6. Textural determinations

Texture parameters were measured using an Instron Compression Tester 4301 (Instron Ltd., Barcelona, Spain) controlled by the BlueHill V2.0 software, with a load cell of 1000 N and crosshead speed of 100 mm/min. The shear strength (N) required to shear the sample was determined using a Warner-Bratzler blade, and the hardness (N), considered as the maximum force required to compress the sample, was determined with a Kramer cell. Six measurements were taken for sample in beef carpaccio with a Warner-Bratzler blade and a Kramer cell, respectively, at 0 h, 7, 15 and 30 d after pressurization.

2.7. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) by means of SPSS program Win 12.0 software (SPSS Inc., Chicago, IL), with treatment and time of storage as main effects. Significant differences between means were assessed by Tukey test with a confidence interval of 95 %.

3. Results and Discussion

3.1. Inactivation of total viable counts (TVC)

TVC in beef carpaccio treated with HHP at 450 MPa, the LP system, ALF, and their combinations, and stored under refrigeration at 8 ºC during 30 d are shown in Table 1. According to the analysis of variance, treatments and storage time had significant ($P<0.001$) effects on TVC counts.

Initial TVC in non-treated beef carpaccio were 6.81 log cfu/g. Immediately after pressurization, reductions in TVC were 3.55 log cfu/g at 450 MPa for 5 min. Levels of TVC decreased 3.28 and 3.24 log cfu/g with the LP system and ALF, respectively. Reductions of 3.82 and 3.69 log cfu/g in TVC were achieved at 450 MPa combined with the LP system or ALF, respectively.

During the storage under refrigeration at 8 ºC, TVC increased by 1.46 log cfu/g in non-treated samples. It is known that storage of meat with restricted oxygen supply, e.g. vacuum-packaging, restricts pseudomonads and allows the growth of facultative anaerobes, such as lactobacilli and other lactic acid bacteria (LAB) (Nottingham, 1982). The increase of TVC was also observed in all treated samples after 30 d at 8 ºC, although TVC were at lower levels than in non-treated beef carpaccio. Combined treatments between HHP and the LP system or ALF were the most effective treatments, achieving similar reductions of 3.75 and 3.73 log cfu/g, respectively, at the end of storage. In the present work, a recovery of sublethally injured TVC levels was observed in non-treated and treated beef carpaccio after 30 d at 8 ºC, in accordance with previous results reported by De Alba, Bravo, & Medina (2012). Levels of TVC in non-treated samples were higher than 6 log cfu/g at the begining of the assay (0 h). According to Garriga, Grébol, Aymerich, Monfort, & Hugas (2004), this value could be attributed to inappropiate slaughterhouse operations, handling or chilled storage before processing. In cured beef carpaccio treated at 400 MPa for 5 min at 20 ºC (Vaudagna et al., 2012) aerobic total counts, pshycrotrophs and LAB were reduced by 2.6, 3.9 and 2.9 log cfu/g, respectively, immediately after

pressurization. On the other hand, Szerman et al. (2011) observed reductions of 3.79 log cfu/g in total counts in thawed beef carpaccio treated at 400 MPa for 5 min at 5 ºC, immediately after treatment. According to Realini et al. (2011), high pressure treatments at 400 and 600 MPa for 6 min at -35 and -15 ºC applied in cured pork carpaccio were not able to completely eliminate LAB and psychrotrophs. Counts at 41 d showed that cells were able to recover during storage at 2 ºC, thus overcoming the sublethal damage induced by HHP. Kennedy et al. (2000) evaluated the degree of inhibition of the LP system of natural microbial populations of microorganisms present in ground beef incubated at room temperature. In all cases, bacteria counts increased in the control samples over the 4 h incubation time, and in general, the growth of the microbial populations was inhibited by the LP system. According to Elliot et al. (2004), at temperatures assayed (12 to -1º C over 1 week and held at -1º C for 4 weeks), the LP system did not inhibit the growth of native LAB in beef meat. In the present work, an increase of TVC levels was detected in samples treated with the LP system, applied individually, at the end of storage. It could be probably due to the growth of LAB (Lucquin, Zagorec, Champomier-Vergès, & Chaillou, 2012). Colak, Hampikyan, Bingol, & Aksu (2008) reported significant ($P<0.05$) reductions in TVC in Turkish-style meatballs by LF, applied alone or in combination with nisin and Montiel et al. (2012) observed that HHP at 450 MPa for 10 min in combination with the LP system, delayed the spoilage of smoked salmon, maintaining TVC below the detection limit during 35 d at 5 ºC.

3.2. pH and a_w

Values of pH and a_w in cured beef carpaccio stored at 8 ºC during 30 d are shown in Table 2. According to the analysis of variance, treatments and storage time had significant ($P<0.001$) effects on pH and a_w throughout refrigerated storage.

Immediately after pressurization, an increase ($P<0.05$) of pH values was observed in samples treated at 450 MPa for 5 min, being attributed to protein denaturation or loss of protons caused by pressurization treatment (Marcos, Aymerich, & Garriga,

2005). ALF applied alone or in combination with HHP hardly affected pH values at 0 h. In accordance with our results, Sammel, Claus, Greaser, & Lucey (2007) did not observe any effect of lactoferrin on pH in cooked ground turkey samples. In the present work, immediately after pressurization, carpaccio treated with HHP and the LP system showed a slight decrease ($P<0.05$) in pH values. After 30 d at 8 ºC, both non-treated samples and those treated with HHP, the LP system, or ALF applied individually, showed a decrease ($P<0.05$) of pH values regarding 0 h, probably due to the growth of LAB (Lucquin et al., 2012). In pressurized samples treated with the antimicrobials, values of pH were kept at the same level than at 0 h, showing a higher effectiveness of the combinations to control the native microbial populations in cured beef carpaccio.

Values of a_w increased significantly ($P<0.05$) in samples treated with HHP, applied alone or in combination with the LP system or ALF, immediately after pressurization. According to Bertram, Whittaker, Shorthose, Andersen, & Karlsson (2004), high pressure treatment alters the interactions between muscle proteins and water, which changes the water characteristics of meat. Szerman et al. (2011) observed that the application of HHP treatment to frozen carpaccio samples minimized myofibrillar protein denaturation, reducing water loss during pressurization. In the present work, no significant influence on a_w values was detected with ALF applied individually, after pressurization. Our results are in accordance with Colak et al. (2008), so they did not observe any effect of LF on a_w values in Turkish-style meatball samples. After 30 d at 8 ºC, values of a_w decreased ($P<0.05$) in all samples, although maximum differences were only of 0.02 units.

3.3. Color

Changes in instrumental color characteristics (L^*, a^* and b^*) of beef carpaccio are shown in Table 3. When HHP was applied alone or in combination with antimicrobials, L^* values were significantly ($P<0.05$) higher in treated than in control samples immediately after pressurization. This whitening effect has been explained by

molecular changes, mainly by denaturation of myoglobin and modification of the porphyrin ring (Carlez, Veciana-Nogués, & Cheftel, 1995). Although HHP increases *L** in fresh meat (Schenkova et al. 2007; McArdle, Marcos, Kerry, & Mullen, 2010; Marcos, Kerry & Mullen, 2010), changes in cured meat products are lower and acceptable from an organoleptic point of view (De Alba et al. 2012) and, according to Ferrini, Comaposada, Arnau, & Gou (2012), depending on the water content and a_w value of the products. A decrease ($P<0.05$) of lightness was observed in samples treated with ALF but not with the LP system. After 30 d at 8 ºC, values of *L** were attenuated and no significant differences were detected in beef carpaccio among treatments. According to Sammel et al. (2007), lactoferrin (30-5000 ppm) increased or decreased pink color depending on its concentration in cooked ground turkey without added sodium nitrite or nicotinamide. Lactoferrin at 30 ppm increased ($P<0.05$) *L** compared to the control when sodium nitrite or no pink-color-generating ligand was added. Montiel et al. (2012) observed that *L** values increased by HHP and the LP system treatments, applied individually or combined, resulting in a brighter and less transparent appearance of smoked salmon.

Redness (*a**) was slightly higher ($P<0.05$) in samples treated with ALF and lower ($P<0.05$) with HHP combined with the LP system, immediately after pressurization. The individual application of the LP system did not affect negatively redness values of cured beef carpaccio. In accordance with Sammel et al. (2007), redness was increased by 30 ppm lactoferrin and decreased by 5000 ppm lactoferrin in beef carpaccio prepared without added sodium nitrite or nicotinamide ($P<0.05$), compared to control samples. According to these authors, these differences could be related to iron sequestering or interactions of lactoferrin with the heme ring of pigments or pink-color-generating ligands.

After 30 d at 8 ºC, *a** values slightly decreased in non-treated samples and in those treated with ALF, although this decrease was higher than the observed in control carpaccio. However, in samples where pressurization was applied alone or in combination with ALF, *a** values increased ($P<0.05$). On the other hand, a slight

decrease ($P<0.05$) of redness was observed in samples where the LP system was applied alone or combined with HHP, regarding non-treated samples, at the end of storage. Montiel et al. (2012) observed a decrease ($P<0.05$) of a^* values in smoked salmon treated with the LP system, applied alone or in combination with 250 MPa and 450 MPa for 10 min, after 35 d at 5 ºC.

Immediately after pressurization, yellowness (b^*) was not affected by treatments. After 30 d at 8 ºC, a decrease ($P<0.05$) of b^* was observed with most of treatments, although yellowness slightly increased ($P<0.05$) in samples treated at 450 MPa for 5 min, applied individually. However, b^* did not change in samples treated with HHP combined with ALF. According to Sammel et al. (2007), lactoferrin did not affect yellowness of cooked ground turkey. On the other hand, the LP system added to smoked salmon, alone or in combination with HHP at 250 MPa for 10 min decreased ($P<0.05$) yellowness, immediately after pressurization (Montiel et al. 2012).

The color of cured meat products is mainly due to the presence of nitrosylmyoglobin, resulting from the reaction of nitric oxide (from sodium nitrite or sodium nitrate) with myoglobin. In the present work, any negative effect on color characteristics was observed with the individual application of the LP system on cured beef carpaccio. The generation *in situ* of H_2O_2 by added glucosa oxidase and glucose did not produce a detrimental effect on nitrosylmyoglobin. On the other hand, frozen conditioning of carpaccio previous to HHP treatments reduced the harmful effects of high pressure on chromatic parameters (L^*, a^* and b^*) (Szerman et al. 2011). This could be due to a minimization of the denaturation of sarcoplasmic and myofibrillar proteins.

3.4. Texture

Texture parameters of pressurized and control beef carpaccio are given in Table 4. No significant differences in shear strength values (N) were observed immediately after pressurization, except in samples treated at 450 MPa combined with the LP system, where an increase ($P<0.05$) of this texture property was detected. After 30 d at 8 ºC, shear strength values increased ($P<0.05$) with antimicrobials and were attenuated with HHP combined with the LP system, regarding 0 h. Szerman et al. (2011) observed that shear force values of frozen or thawed beef carpaccio treated by HHP were higher than their respective controls, although those differences were not significant. According to Vaudagna et al. (2012), treatments at 400 MPa and 650 MPa for 1 and 5 min at 20 ºC, respectively, applied to cured beef carpaccio produced a significative effect ($P<0.05$) on shear force values, achieving the lowest and the highest values with those treatments.

Hardness (N) was not significantly affected by treatments, immediately after pressurization. A slight increase ($P<0.05$) of hardness was observed in non-treated samples at the end of storage. After 30 d at 8 ºC, hardness of cured beef carpaccio slightly decreased ($P<0.05$) with HHP, applied individually, except for samples treated with the LP system that exhibited an increase of this value.

4. Conclusions

Pressurization at 450 MPa for 5 min combined the LP system or with ALF were the most effective treatments to achieve the lowest levels of TVC in cured beef carpaccio after 30 d at 8 ºC, although TVC were lower in all treated samples compared with those non-treated, at the end of storage. Treatments maintained TVC lower than initial counts in carpaccio during the 30 d of refrigeration under mild temperature abuse conditions. Color and texture of cured beef carpaccio were slightly modified by treatments although the quality of this product was not negatively affected. Further

studies would be necessary to understand the effect of the LP system, ALF, HHP and their combinations on the characteristics of meat products, such as color and texture.

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Table 1. Levels (log cfu/g) of total viable counts (TVC) in beef carpaccio treated with HHP at 450 MPa, the LP system, ALF, and their combinations, during 30 d at 8 ºC.

	0 h	1 d	7 d	15 d	30 d
Control	6.81 ± 0.34 ^{dA}	7.05 ± 0.30 ^{eB}	7.64 ± 0.24 ^{eC}	7.74 ± 0.25 ^{dC}	8.27 ± 0.12 ^{eD}
450 MPa	3.26 ± 0.22 ^{bB}	3.11 ± 0.09 ^{bA}	3.44 ± 0.13 ^{cC}	4.17 ± 0.50 ^{cD}	6.29 ± 0.12 ^{dE}
LPS	3.53 ± 0.19 ^{cA}	3.78 ± 0.09 ^{dB}	3.73 ± 0.03 ^{dB}	3.46 ± 0.09 ^{bA}	5.14 ± 0.15 ^{bC}
ALF	3.57 ± 0.03 ^{cAB}	3.52 ± 0.18 ^{cA}	3.66 ± 0.07 ^{dB}	3.60 ± 0.09 ^{bAB}	5.41 ± 0.02 ^{cC}
450 MPa + LPS	2.99 ± 0.14 ^{aB}	2.95 ± 0.09 ^{aB}	2.88 ± 0.12 ^{aAB}	2.75 ± 0.20 ^{aA}	4.52 ± 0.07 ^{aC}
450 MPa + ALF	3.12 ± 0.09 ^{abA}	3.15 ± 0.04 ^{bA}	3.10 ± 0.10 ^{bA}	3.33 ± 0.48 ^{bA}	4.54 ± 0.22 ^{aB}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 2. Values of pH and a_w in beef carpaccio treated with HHP at 450 MPa, the LP system, ALF, and their combinations, during 30 d at 8 ºC.

		0 h	1 d	7 d	15 d	30 d
pH	Control	5.48 ± 0.13 ^{abD}	5.36 ± 0.05 ^{aBC}	5.41 ± 0.04 ^{dCD}	5.28 ± 0.06 ^{cAB}	5.22 ± 0.05 ^{bA}
	450 MPa	5.59 ± 0.14 ^{cC}	5.31 ± 0.06 ^{aAB}	5.24 ± 0.02 ^{bA}	5.21 ± 0.02 ^{abA}	5.39 ± 0.10 ^{cB}
	LPS	5.39 ± 0.09 ^{abC}	5.44 ± 0.05 ^{bD}	5.09 ± 0.02 ^{aA}	5.17 ± 0.17 ^{aB}	5.09 ± 0.01 ^{aA}
	ALF	5.50 ± 0.06 ^{bcC}	5.48 ± 0.12 ^{bcC}	5.22 ± 0.01 ^{bB}	5.23 ± 0.03 ^{bcB}	5.05 ± 0.04 ^{aA}
	450 MPa + LPS	5.38 ± 0.01 ^{aB}	5.52 ± 0.04 ^{cC}	5.08 ± 0.07 ^{aA}	5.34 ± 0.05 ^{dB}	5.35 ± 0.02 ^{cB}
	450 MPa + ALF	5.49 ± 0.02 ^{bcC}	5.58 ± 0.03 ^{dD}	5.34 ± 0.13 ^{cA}	5.42 ± 0.10 ^{eB}	5.49 ± 0.05 ^{dC}
a_w	Control	0.921 ± 0.001 ^{aBC}	0.922 ± 0.002 ^{aC}	0.918 ± 0.001 ^{aA}	0.919 ± 0.001 ^{aAB}	0.919 ± 0.001 ^{bA}
	450 MPa	0.926 ± 0.000 ^{bC}	0.926 ± 0.002 ^{bC}	0.922 ± 0.003 ^{bB}	0.922 ± 0.001 ^{bB}	0.919 ± 0.001 ^{bA}
	LPS	0.924 ± 0.002 ^{abB}	0.923 ± 0.002 ^{aB}	0.921 ± 0.005 ^{bA}	0.924 ± 0.001 ^{bcB}	0.920 ± 0.000 ^{bcA}
	ALF	0.921 ± 0.001 ^{aC}	0.922 ± 0.001 ^{aC}	0.922 ± 0.001 ^{bC}	0.919 ± 0.003 ^{aB}	0.915 ± 0.002 ^{aA}
	450 MPa + LPS	0.929 ± 0.001 ^{cC}	0.928 ± 0.001 ^{cC}	0.924 ± 0.001 ^{cB}	0.925 ± 0.002 ^{cB}	0.921 ± 0.001 ^{cA}
	450 MPa + ALF	0.934 ± 0.002 ^{dB}	0.933 ± 0.002 ^{dB}	0.934 ± 0.001 ^{dB}	0.933 ± 0.002 ^{dB}	0.924 ± 0.001 ^{dA}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 3. Color parameters in beef carpaccio treated with HHP at 450 MPa, the LP system, ALF, and their combinations, during 30 d at 8 ºC.

		0 h	7 d	15 d	30 d
*L**	Control	34.32 ± 2.90 ^{bA}	32.24 ± 3.82 ^{aA}	31.85 ± 1.32 ^{aA}	33.33 ± 3.50 ^{aA}
	450 MPa	37.31 ± 1.71 ^{cA}	37.03 ± 1.75 ^{bcA}	38.34 ± 3.05 ^{cA}	36.22 ± 2.22 ^{aA}
	LPS	34.79 ± 1.71 ^{bA}	35.63 ± 3.07 ^{bcA}	34.18 ± 3.87 ^{abA}	33.49 ± 2.47 ^{aA}
	ALF	30.92 ± 1.24 ^{aA}	34.12 ± 2.05 ^{abBC}	32.99 ± 2.91 ^{abAB}	35.80 ± 2.59 ^{aC}
	450 MPa + LPS	38.22 ± 2.47 ^{cA}	37.56 ± 1.91 ^{cA}	35.86 ± 1.60 ^{bcA}	36.06 ± 2.83 ^{aA}
	450 MPa + ALF	38.00 ± 2.72 ^{cB}	35.54 ± 1.56 ^{bcA}	35.47 ± 0.97 ^{bcA}	35.40 ± 1.44 ^{aA}
*a**	Control	19.58 ± 1.98 ^{abAB}	20.39 ± 3.90 ^{bB}	18.36 ± 2.25 ^{bcAB}	17.34 ± 2.93 ^{abA}
	450 MPa	17.61 ± 1.96 ^{abAB}	18.01 ± 1.61 ^{abB}	15.78 ± 2.58 ^{abA}	20.29 ± 1.84 ^{bC}
	LPS	17.73 ± 3.22 ^{abA}	17.97 ± 3.15 ^{abA}	19.38 ± 3.53 ^{cA}	16.37 ± 4.96 ^{aA}
	ALF	20.32 ± 2.03 ^{bB}	17.47 ± 2.53 ^{abA}	19.80 ± 3.36 ^{cAB}	17.41 ± 2.69 ^{abA}
	450 MPa + LPS	17.11 ± 2.78 ^{aA}	16.81 ± 2.65 ^{aA}	14.70 ± 2.06 ^{aA}	16.22 ± 2.79 ^{aA}
	450 MPa + ALF	17.69 ± 2.61 ^{abAB}	16.58 ± 2.76 ^{aA}	18.06 ± 1.52 ^{bcAB}	19.55 ± 3.22 ^{abB}
*b**	Control	7.19 ± 1.08 ^{aB}	9.16 ± 3.68 ^{bC}	6.33 ± 1.24 ^{aAB}	4.70 ± 0.97 ^{aA}
	450 MPa	7.61 ± 0.82 ^{aAB}	8.08 ± 1.43 ^{abB}	6.04 ± 1.69 ^{aA}	8.30 ± 2.07 ^{cB}
	LPS	6.82 ± 1.35 ^{aAB}	7.52 ± 2.07 ^{abB}	7.16 ± 2.31 ^{aAB}	5.10 ± 2.37 ^{aA}
	ALF	6.73 ± 1.31 ^{aB}	6.62 ± 1.53 ^{aB}	7.09 ± 1.63 ^{aB}	4.90 ± 1.35 ^{aA}
	450 MPa + LPS	7.18 ± 1.27 ^{aB}	7.23 ± 1.42 ^{abB}	6.48 ± 2.02 ^{aAB}	5.45 ± 1.17 ^{abA}
	450 MPa + ALF	7.44 ± 1.38 ^{aA}	7.43 ± 1.54 ^{abA}	7.35 ± 1.26 ^{aA}	7.28 ± 2.38 ^{bcA}

Values are the mean ± SD.

Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.

Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 4. Texture properties of beef carpaccio treated with HHP at 450 MPa, the LP system, ALF, and their combinations, during 30 d at 8 ºC.

		0 h	7 d	15 d	30 d
Shear force (N)	Control	23.22 ± 6.65 ^{aA}	26.01 ± 7.27 ^{aA}	22.85 ± 2.41 ^{aA}	25.54 ± 9.01 ^{aA}
	450 MPa	30.36 ± 7.87 ^{aA}	26.15 ± 8.55 ^{aA}	33.37 ± 5.77 ^{cA}	29.81 ± 6.39 ^{aA}
	LPS	28.37 ± 6.35 ^{aA}	34.84 ± 3.66 ^{aB}	25.26 ± 2.95 ^{abA}	40.43 ± 8.18 ^{bB}
	ALF	23.84 ± 8.80 ^{aA}	30.23 ± 7.83 ^{aAB}	25.56 ± 6.25 ^{abAB}	32.69 ± 7.06 ^{abB}
	450 MPa + LPS	43.92 ± 14.74 ^{bB}	29.99 ± 9.79 ^{aA}	28.80 ± 4.45 ^{bcA}	30.32 ± 7.76 ^{aA}
	450 MPa + ALF	30.28 ± 4.94 ^{aA}	35.47 ± 11.46 ^{aA}	31.83 ± 6.76 ^{cA}	32.18 ± 8.08 ^{abA}
Hardness (N)	Control	121.11 ± 31.47 ^{aAB}	91.31 ± 14.33 ^{aA}	100.97 ± 10.98 ^{aA}	137.03 ± 56.82 ^{abB}
	450 MPa	111.52 ± 41.58 ^{aAB}	154.83 ± 35.44 ^{cB}	127.03 ± 14.81 ^{abAB}	103.32 ± 34.51 ^{aA}
	LPS	107.95 ± 24.89 ^{aA}	115.80 ± 17.24 ^{abA}	128.59 ± 33.53 ^{abAB}	163.81 ± 13.03 ^{bB}
	ALF	91.96 ± 14.24 ^{aA}	119.10 ± 23.62 ^{abA}	119.78 ± 27.00 ^{abA}	106.00 ± 23.68 ^{aA}
	450 MPa + LPS	117.88 ± 19.07 ^{aA}	132.72 ± 15.59 ^{bcA}	114.91 ± 14.49 ^{abA}	131.62 ± 38.16 ^{abA}
	450 MPa + ALF	103.60 ± 30.99 ^{aA}	130.52 ± 15.56 ^{bcAB}	154.39 ± 22.68 ^{bB}	108.36 ± 33.66 ^{aA}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$

Highlights

- Combined treatments between HHP at 450 MPa for 5 min and the LP system or ALF were the most effective in the inactivation of total viable counts in beef carpaccio.

- The generation *in situ* of H_2O_2 by added glucose oxidase and glucose did not affect negatively the color of beef carpaccio.

- Changes in texture of beef carpaccio were slight.

3.7. Combined treatments between high hydrostatic pressure and bacteriocins on the inactivation of *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* O157:H7 in cured beef carpaccio. *International Journal of Food Microbiology* (Manuscrito en preparación)

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Combined treatments between high hydrostatic pressure and bacteriocins on the inactivation of *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* O157:H7 in cured beef carpaccio

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Running title: High-pressure and bacteriocins on *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 in beef carpaccio.

Abstract

The antimicrobial effect of high hydrostatic pressure (HHP) treatments, bacteriocins and their combinations on *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* O157:H7 was investigated in cured beef carpaccio stored at 8 ºC during 30 d. Immediately after HHP, bacteriocins reduced *Listeria* counts by 0.1 to 1.5 log units, being commercial pediocin the most effective against *L. monocytogenes*. On the contrary, the survival of *S*. Enteritidis or *E. coli* was not affected by bacteriocins. The effect registered with the combination of HHP with enterocins A and B or commercial nisin was additive on *L. monocytogenes*, and similar activity was detected on *Salmonella* in carpaccio treated with HHP combined with enterocins A and B or pediocin PA-1. However, the combination of HHP with enterocins A and B, pediocin PA-1 or commercial nisin produced a synergistic antimicrobial effect on *E. coli* counts achieving inactivation rates approximately 2 log units higher than the obtained with the sum of the individual antimicrobial activities. Lightness (*L**) increased with pressurization and redness (*a**) or yellowness (*b**) decreased, whereas minor changes were observed in textural properties, although shear strength tended to increase at the end of storage.

Keywords: *L. monocytogenes*, *S.* Enteritidis, *E. coli* O157:H7, beef carpaccio, high pressure, bacteriocins.

1. Introduction

Nowadays, there is an upward trend among consumers to minimally processed, with fresh appearance, healthier and safe ready-to-eat (RTE) products. A wide variety of RTE meat products are available in the market and have a significant position. Beef carpaccio, prepared from raw or cured pieces of meat usually frozen, is sliced, packaged under vacuum or modified atmospheres and marketed at refrigeration temperatures. Raw beef can be contaminated with pathogenic bacteria, such as *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli* O157:H7. According to Rhoades et al. (2009), *Salmonella* was found in 3.8% of raw (mostly minced) beef and *L. monocytogenes* and *E. coli* O157:H7 were detected on 10% of raw beef products, around the world. For these reasons, it would be very useful to optimize the most convenient preservation technologies to improve the safety and extend the shelf-life of beef carpaccio.

Bacteriocins produced by lactic acid bacteria (LAB) are small, heat-stable cationic ribosomally peptides with a wider spectrum of inhibition (Cotter et al., 2005). Nisin is a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis* (Ross et al., 2003) with a broad spectrum of activity against most Gram-positive bacteria and also inhibits the outgrowth of *Bacillus* spp. and *Clostridium* spp. spores (Savadogo et al., 2006). It is the only bacteriocin approved to be used as a food additive (E234) (European Parliament and of the Council, 1995) and it has generally regarded as safe (GRAS) status by FDA (1988), with many applications in foods (Delves-Broughton, 2005). Nisin forms pores on the plasma membrane that disrupt the proton motive force, and causes depletion of essential molecules for the target bacterium and the inhibition of the peptidoglycan synthesis by interacting with the peptidoglycan precursor lipid II (Wiedemann et al., 2001). However, it has been shown that the application of nisin in meat products faces several limitations derived from its interaction with phospholipids emulsifiers and other food constituents (Aasen et al., 2003). Pediocin PA-1, produced by *Pediococcus acidilactici*, acts on the cell

membrane by forming pores, which leads to the dissipation of the electrochemical gradient and cellular death (Deegan et al., 2006). Pediocin PA-1 is active against foodborne pathogens including *L. monocytogenes*, *Staphylococcus aureus*, *C. perfringens*, and other bacteria closely related to the producer strain *P. acidilactici* PA-1 (Bhunia et al., 1988; González and Kunka, 1987; Farber and Peterkin, 1991). Pediocin PA-1 and nisin are commercially available, although the commercial preparation based on pediocin PA-1 is not approved as a food additive (Gálvez et al., 2008). Enterocins A and B are produced by some strains of *Enterococcus faecium* and their antilisterial activity has been shown in meat homogenates and applied experimentally as ingredients in several meat products (Aymerich et al., 2000; Garriga et al., 2002).

However, the efficacy of bacteriocins in foods will greatly depend on a number of food-related factors, food microbiota or the target bacteria (Gálvez et al., 2007) and can be limited if applied alone. For this reason, the combination of bacteriocins with high hydrostatic pressure (HHP) can be effective against bacterial inactivation, at once to decrease the intensity of treatments and extending the shelf-life of the product without compromising microbial safety.

Although many studies have been conducted on the use of HHP combined with bacteriocins in different meat products (Aymerich et al., 2005; Jofré et al., 2007, 2008a, 2008b, 2008c, 2009; Garriga et al., 2002; Chung et al., 2005; Hereu et al., 2012; De Alba et al., 2013; Marcos et al., 2008a, 2008b; Yuste et al., 2002), to our knowledge, the effect of this hurdle technology on the inactivation of *L. monocytogenes*, *S*. Enteritidis and *E. coli* O157:H7 in cured beef carpaccio stored under mild temperature abuse conditions at 8 ºC has not been investigated.

The aim of this work was to evaluate the effect of HHP in combination with bacteriocins from supernatants of lactic acid bacteria (LAB) cultures, such as pediocin PA-1, nisin Z, and enterocins A and B, and two commercial preparations based on nisin or pediocin, on the inactivation of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 in cured beef carpaccio stored during 30 d at 8 ºC.

2. Materials and methods

2.1. Bacterial cultures

Listeria monocytogenes INIA H66a (from the INIA Culture Collection, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain), *Salmonella enterica* subsp. *enterica* serovar Enteritidis strains CECT 4155, CECT 4300 and CECT 4396, and *Escherichia coli* O157:H7 CECT 4972 (from the Spanish Type Culture Collection, Valencia, Spain) were grown over-night in trypticase soy yeast extract broth (TSYEB) (Biolife, Milano, Italy) at 37 ºC for 18 h to inoculate carpaccio samples. Nisin-Z producing *Lactococcus lactis* subsp. *lactis* INIA TAB 26, *E. faecium* INIA TAB 7 producing enterocins A and B (both from the INIA Culture Collection) and *P. acidilactici* 347 (from Universidad Complutense, Madrid, Spain) were grown in Man Rogosa & Sharpe (MRS) broth (Biolife) at 30 ºC for 18 h to be used as bacteriocinogenic cultures. *L. innocua* BL86/26 (from Università Cattolica del Sacro Cuore, Italy) was grown in TSYEB at 37 ºC for 18 h and *Lactobacillus buchneri* St2A (from NIZO Food Research, Ede, The Netherlands) was grown at 1% in MRS broth at 30 ºC for 18 h. Both microorganisms were used as indicators strains. All strains were kept at -80 ºC in a suitable medium for each microorganism with 30% glycerol.

2.2. Bacteriocin assay

Bacteriocinogenic cultures were centrifugated at 10.000 g during 15 min at 4 ºC in a centrifuge 5810R (Eppendorf, Hamburg, Germany). Supernatants were adjusted to pH 6.0 with 1 N NaOH and filtered by passing through a 0.22 µm-pore-size cellulose acetate filters (Millipore, Bedford, MA). A volume of 25 µL of each supernatant was placed in triplicate into wells (5 mm diameter) made in pour plates of TSYEA and MRS agar inoculated with 0.1% of 18 h cultures of *L. innocua* BL86/26 and *L. buchneri* St2A as indicator microorganisms. After incubation at 37 ºC or 30 ºC for 24 h, respectively, the diameter of the zone of growth inhibition was measured and bacteriocin activity was expressed in mm.

2.3. Sample preparation

Cured beef carpaccio was obtained in a retail butcher in Madrid (Spain) and kept frozen until used. Slices were aseptically cut into 10 g pieces and inoculated by spreading 100 µL of diluted over-night cultures of *L. monocytogenes*, *S*. Enteritidis and *E. coli* O157:H7. The pathogens were inoculated separately in order to obtain independent batches for each one. Non-inoculated samples were also prepared to determine total viable counts (TVC), pH, a_w, texture and color. Beef carpaccio was sliced into 2-mm-thick slices to determine texture and color characteristics. Inoculated and non-inoculated samples were individually vacuum-packed in double bags of BB325 (Cryovac Sealed Air Corporation, Milan, Italy) and held at 4 ºC for 18 h until applying the individual or combined treatments.

2.4. Addition of biopreservatives

Commercial nisin was prepared in 0.02 M HCl (0.02 g/ml). A commercial product based on pediocin was dissolved in distilled-deionized water. Both biopreservatives were added on the surface of cured beef carpaccio at 100 IU/g and 0.6% for nisin and pediocin, respectively.

2.5. HHP processing

High pressure treatments were performed in a prototype ACIP 6000 (ACB, Nantes, France) of 3.5 L capacity and 600 MPa maximum working pressure. Water was used as pressure transmitting fluid. Samples were pressurized at 450 MPa for 5 min at 12 ºC. After pressurization, all samples were stored at 8 ºC during 30 d. Two independent trials were carried out.

2.6. Microbiological analysis

Samples were analyzed immediately after HHP treatment and after 1, 3, 7, 15 and 30 d. Samples (10 g) were homogenized in 90 ml of 0.1% (wt/vol) sterile peptone water using a homogenizer (IUL, Barcelona, Spain). Decimal dilutions were prepared in the same sterile solution. *Listeria*, *Salmonella* and *E. coli* populations were determined on duplicate plates of Chromagar Listeria (CHROMagar, Paris, France), Salmonella Shigella agar (SSA, Scharlab S.L., Barcelona, Spain) and Violet Red Bile Agar (VRBA, Oxoid Ltd., Basingstoke, Hampshire, England), respectively, incubated at 37 ºC for 24 h. TVC were determined immediately after HHP treatments and after 1, 7, 15 and 30 d on duplicate plates of tryptic soy agar (TSA) (Biolife) incubated at 30 ºC for 72 h.

2.7. pH and a_w

Beef carpaccio samples (5 g) were homogenized with 45 mL of distilled water in stomacher bags for 90 s in a homogenizer and pH determinations were taken using a pH-meter (model GPL22, Crison Instruments, Barcelona, Spain). Water activity (a_w) was measured in carpaccio using the AquaLab Series 3 equipment (Decagon Devices, Inc., Pullman, WA, EE.UU.). Samples were taken at 0 h, 1, 7, 15 and 30 d after pressurization. Two measurements were carried out per sample.

2.8. Color measurements

Surface color of beef carpaccio was measured six times per sample using a Minolta CM 700d Chromometer (Minolta Camera Co., Osaka, Japan). *L** (lightness), *a** (redness) and *b** (yellowness) were determined at 0 h, 7, 15 and 30 d after pressurization.

2.9. Textural determinations

Texture parameters were measured using an Instron Compression Tester 4301 (Instron Ltd., Barcelona, Spain) controlled by the BlueHill V2.0 software, with a load

cell of 1000 N and crosshead speed of 100 mm/min. Hardness (N), considered as the maximum force required to compress the sample, was determined with a Kramer cell and the shear strength (N) required to shear the sample was examined using a Warner-Bratzler blade. Six measurements of each texture parameter were taken per sample at 0 h, 7, 15 and 30 d after pressurization.

2.10. Statistical analysis

Data were subjected to analysis of variance (ANOVA) of SPSS program Win 12.0 software (SPSS Inc., Chicago, IL). Significant differences between means were assessed by Tukey test ($P<0.05$).

3. Results and Discussion

3.1. Effect of treatments on Listeria monocytogenes *population*

Levels (log cfu/g) of *L. monocytogenes* in cured beef carpaccio subjected to HHP at 450 MPa for 5 min, bacteriocins and their combinations, stored at 8 ºC during 30 d are shown in Table 1. *L. monocytogenes* mean initial counts in inoculated beef carpaccio were 6.67 log cfu/g. Immediately after pressurization, reductions of *L. monocytogenes* were 1.09 log cfu/g in pressurized carpaccio at 450 MPa. Bacteriocins applied individually reduced counts by 0.1 to 1.5 log units, being commercial pediocin, the most effective bacteriocin against *Listeria*. Most food applications of this bacteriocin have resulted in reductions of 1 to 3 log units in populations of potential foodborne pathogens, since factors such as bacteriocin binding food components, or poor solubility or stability in the food substrate may diminish pediocin activity (Muriana et al., 1996). On the other hand, nisin Z was the less effective bacteriocin against *L. monocytogenes* inoculated in beef carpaccio. The mechanism of *L. monocytogenes* resistance to nisin seems to be correlated with changes in membrane fatty acid composition, cell wall structure and requirements for divalent cations (Crandall and Montville, 1998). The inactivation achieved at 450 MPa combined with

enterocins A and B or commercial nisin was 0.26 log units higher than the sum of log reductions obtained with each single treatment and as a result, a slight synergistic antimicrobial effect was observed. The reductions of *L. monocytogenes* counts in pressurized samples combined with pediocin PA-1, nisin Z or commercial pediocin were 1.62, 1.18 and 2.48 log units and consequently, an additive antimicrobial effect was registered.

During the storage under refrigeration at 8 ºC, counts of *Listeria* only diminished by 0.27 log cfu/g in non-treated samples, indicating the ability of *L. monocytogenes* to survive in cured beef carpaccio during 30 d at mild temperature abuse conditions, but no growth of the pathogen was observed. Values of water activity (a_w) in the lower limit (0.92) for growth of *L. monocytogenes* in control samples as well as the curing mixture present in this product would avoid the growth of the pathogen. It is known that combined effects of intrinsic factors such as pH, salt, sugar, chemical additives and extrinsic inhibitory barriers such as storage conditions work synergistically to ensure the microbiological safety of foods (Leistner, 2000). In this regard, Rhoades et al. (2013) observed that the growth of *L. monocytogenes* inoculated in marinated beef was completely inhibited by the soy sauced and red wine-based marinades during storage of this product, even at conditions simulating temperature abuse (15 ºC), and additional antimicrobial components could increase the antimicrobial effect of marinades.

In the present work, an additive antimicrobial effect was observed in samples treated at 450 MPa combined with enterocins A and B or commercial nisin at the end of storage period. However, the antimicrobial effect detected with nisin Z or commercial pediocin combined with pressurization was slightly synergistic, achieving reductions 0.26 and 0.36 log units higher than the sum of the effects obtained with individual treatments. In a meat model system, combined treatments between sakacin K, enterocins A and B or pediocin AcH and HHP at 400 MPa for 10 min kept *L. monocytogenes* below or equal to the detection limit ($<10^2$ cfu/g) during 61 d at 4 ºC (Garriga et al., 2002). However, in non-treated samples and in those treated with

HHP and nisin, pathogen survivors recovered rapidly during storage. Combinations of nisin at 200 AU/cm^2 applied directly or through active packaging with HHP at 600 MPa for 5 min on dry-cured ham provided a wide margin of safety in controlling *L. monocytogenes* during the 60 d at 8 ºC (Hereu et al., 2012). The recovery of this pathogen inoculated on sliced cooked ham stored at 6 ºC was delayed after the cold chain break, when HHP was applied at 400 MPa for 10 min in combination with enterocins A and B (Marcos et al., 2008b), although the presence of the bacteriocin was not effective enough to prevent the growth of the pathogen. However, the combination of 400 MPa for 10 min and enterocin LM-2 at 2560 AU/g kept *L. monocytogenes* below 100 cfu/g during 90 d at 4 ºC in sliced cooked ham (Liu et al., 2012).

3.2. Effect of treatments on Salmonella *Enteritidis population*

Counts of *S*. Enteritidis in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, and stored during 30 d at 8 ºC are shown in Table 2. *S*. Enteritidis mean initial counts were 5.76 log units in cured beef carpaccio. Immediately after pressurization, reductions of *S*. Enteritidis were 4.13 log cfu/g in samples treated at 450 MPa for 5 min, showing the high sensitivity of the pathogen to HHP. However, bacteriocins applied individually were not active against *S*. Enteritidis. These results are in accordance with Jofré et al. (2008a) who did not observe reductions in *Salmonella* levels inoculated in sliced cooked ham which contained interleavers with enterocins A and B, sakacin K or nisin, after either 1 day or during 3 months at 6 ºC. It has been shown that bacteriocins such as nisin or pediocin PA-1 fail to kill *Salmonella* unless combined, for example with HHP (Kalchayanand et al., 2004). Nisin and pediocin PA-1 cannot pass across the outer membrane of Gram-negative bacteria, and therefore, they are not active on *Salmonella* (Chalón et al., 2012). However, other authors reported inhibitory activity of bacteriocins against *Salmonella*, such as enterocin P (Kang and Lee, 2005),

enterocin AS-48 (Ananou et al., 2010), plantaricin MG (Gong et al., 2010) or lactococcin BZ (Sahingil et al., 2011).

Immediately after treatments, although an antimicrobial effect was observed with combined treatments, the level of inactivation was not higher than that obtained with pressurization applied individually. It is known that Gram-negative bacteria may become sensitized to bacteriocins upon exposure to hurdles such as HHP that destabilizes the bacterial outer membrane and alters bacterial permeability (Kalchayanand et al., 1994; Hauben et al., 1996; Kato and Hayashi, 1999). However, a synergistic antimicrobial effect higher than 1 log unit was observed after 24 and 72 h on *Salmonella* counts when HHP and commercial nisin were combined.

In the present work, during the storage under refrigeration at 8 ºC, counts of *Salmonella* diminished 0.41 log cfu/g in non-treated samples, indicating that *S*. Enteritidis was able to survive in beef carpaccio stored during 30 d at 8 ºC. However, no growth of the pathogen was observed during the storage, probably due to the antimicrobial activity of the curing ingredients and the low water activity (a_w) values in non-treated samples. Lanciotti et al. (2001) recorded a minimum a_w of 0.948 for the growth of *S*. Enteritidis. Also, the growth of *S*. Enteritidis in raw beef stored at both 5 ºC and 15 ºC was prevented by the marinades applied in the product (Rhoades et al., 2013). In sliced cooked ham the growth of *S*. Enteritidis was inhibited at 4 ºC (Liu et al., 2012), with a slight reduction of 0.92 log units in the pathogen counts during 90 d of storage.

The antimicrobial effect of HHP was maintained during storage, with counts decreasing throughout refrigeration. After 30 d at 8 ºC, levels of *Salmonella* in pressurized carpaccio were lower that the registered in samples treated with combined pressurization and bacteriocins. *Salmonella* was not detected in cooked ham pressurized at 400 MPa during 10 min combined with the application of nisin at 200 AU/cm^2 through interleavers (Jofré et al., 2008a) and no further recovery of the pathogen was observed during 90 d of storage at 6 ºC. With treatments of 600 MPa for 5 min and enterocins A and B, sakacin K or nisin in cooked and dry cured ham,

the antimicrobial effect was higher than the observed in the present work, the pathogen was not detected from day 7 to 57 of storage at 4 ºC and from day 58 to 120 at 15 ºC (Jofré et al., 2008c), being irrespective of the bacteriocin applied, the kind of meat product and the abuse of temperature. Also the combination of 400 MPa for 10 min and enterocin LM-2 (Liu et al., 2012) reduced the counts of *S*. Enteritidis below the detection limit in sliced cooked ham during 90 d at 4 ºC.

3.3. Effect of treatments on Escherichia coli *O157:H7 population*

Survival of *E. coli* O157:H7 in cured beef carpaccio subjected to individual or combined application of HHP and bacteriocins, stored at 8 ºC during 30 d is shown in Table 3. *E. coli* mean initial counts in cured beef carpaccio were 6.23 log cfu/g. Immediately after HHP, reductions of *E. coli* O157:H7 were 2.72 log cfu/g in samples treated at 450 MPa for 5 min, whereas *E. coli* counts were not affected by the individual application of bacteriocins. Similar results were observed in dry-cured ham inoculated with *E. coli* O157:H7 when nisin or pediocin were applied individually (De Alba et al., 2013) and in cooked ham with enterocins A and B, sakacin K, pediocin AcH and nisin against *E. coli* CTC1018 and CTC1023 (Garriga et al., 2002) which decreased by more than 6 log units in cooked ham treated at 400 MPa combined with nisin A. In the present work, *E. coli* cells were sensitized to bacteriocins when combined with HHP, as reported by Kalchayanand et al. (2004).

During the storage at 8 ºC, counts of *E. coli* remained relatively unchanged in non-treated samples, showing the ability of the pathogen to survive in cured beef carpaccio. *E. coli* O157:H7 is able to survive in other meat products such as dry-cured ham (De Alba et al., 2013) or fermented sausages (Holck et al., 2011).

In the present work, after 30 d at 8 ºC, a synergistic antimicrobial effect was observed with combined treatments between HHP and enterocins A and B, pediocin PA-1 or commercial nisin, with inactivation rates approximately 2 log units higher than the achieved with the sum of the individual effects. The combination of commercial pediocin and HHP produced a lower synergistic antimicrobial effect on *E.*

coli counts, achieving reductions of 1.17 log units higher than the sum of individual effects. A slight synergistic antimicrobial effect was also detected with nisin Z combined with 450 MPa for 5 min. According to De Alba et al. (2013), a synergistic antimicrobial effect on *E. coli* O157:H7 inoculated in dry-cured ham treated at 500 MPa combined with nisin was kept during 60 d at 8 ºC.

3.2. Inactivation of total viable counts (TVC)

TVC in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC are shown in Table 4. Initial TVC in non-treated cured beef carpaccio were 7.09 log cfu/g. Immediately after HHP, reductions of 2.14 log cfu/g were achieved at 450 MPa for 5 min. The individual application of enterocins A and B, pediocin PA-1 or nisin Z produced reductions of 0.36, 0.55 or 0.28 log units, respectively. Commercial nisin reduced TVC by 1.18 log units, being the most effective bacteriocin applied individually. The application of commercial pediocin and pressurization produced a synergistic antimicrobial effect with reductions 0.88 log units higher than the sum of the inactivation obtained with each single treatment.

During refrigeration, levels of TVC increased 0.94 log cfu/g in non-treated beef carpaccio, probably due to the growth of lactic acid bacteria mainly present in vacuum-packed beef carpaccio (Lucquin et al., 2012). A recovery of TVC levels was also detected in all treated samples, and only in those where pressurization was applied alone or combined with commercial nisin or pediocin, TVC levels remained lower ($P<0.05$) than in control group. The reductions observed with these treatments were 0.34 and 0.64 log units higher than the sum of the inactivation obtained with each single treatment.

The shelf-life of meat and meat products depends on many factors including the number and types of microorganisms initially present or introduced by cross-contamination and their subsequent growth affected by processing conditions and temperature storage (Borch et al., 1996; Nychas et al., 2008). Shelf-life of meat and meat products may be extended by vacuum packing in plastic materials with low

permeability to oxygen since oxygen restriction will gradually select CO_2-tolerant organisms, being *Lactobacillus* spp., *Leuconostoc* spp. and *Carnobacterium* spp., the predominant microorganisms (Fontana et al., 2006; Jones, 2004). The control of indigenous LAB and also the spoilage bacteria through the addition of bacteriocins directly on the surface of meat products or on packaging, applied individually or combined with HHP has merited attention. Pediocin PA-1, sakacin A and enterocin A were active against lactic acid bacteria (Aymerich et al., 1998), and lactocin 705 was also antagonistic toward lactic acid bacteria and *Brochothrix thermosphacta* when assayed in meat systems (Castellano et al., 2004; Castellano and Vignolo, 2006) and reduced the growth of psychrotrophic microbiota naturally present in fresh bovine meat (Castellano et al., 2008). On the contrary, Garriga et al. (2002) found that the combined treatment between HHP at 400 MPa for 10 min at 17 ºC and sakacin K or pediocin AcH did not prevent the growth of slime-producing lactic acid bacteria in cooked ham during 60 d of storage at 4 ºC, whereas no recovery of survivors was observed in samples treated with HHP and nisin. Enterocin LM-2 was very effective to extend the shelf-life of sliced cooked ham to above 90 d of storage at 4 ºC and could keep aerobic, psychrotrophic and lactic acid bacteria below the minimal spoilage level (10^4 cfu/g) throughout the storage (Liu et al., 2012) when HHP at 400 MPa was applied in combination with the bacteriocin. The combination of HHP and bacteriocins also significantly reduced the initial number of *Enterobacteriaceae* compared to the control and the highest decrease was observed at 400 MPa for 10 min combined with 2560 AU/g enterocin LM-2. According to Pal et al. (2008), the increase of shelf-life of sliced uncured turkey breast and cured ham subjected to HHP in combination with bacteriocins was due to an increase in the duration of lag phase of bacteria after application of HHP and bacteriocins treatment.

3.3. pH and a_w

Values of pH and a_w in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC are shown in Tables 5 and 6,

respectively. Immediately after pressurization, values of pH decreased significantly ($P<0.05$) in samples treated with pediocin PA-1 or commercial nisin applied individually or in combination with HHP. During refrigeration, values of pH decreased both in non-treated and treated samples, fact that can be attributed to the activity of lactic acid bacteria in beef carpaccio (Lucquin et al., 2012; De Alba et al., 2012; Sánchez-Basurto et al., 2012).

Values of a_w increased significantly ($P<0.05$) in samples treated at 450 MPa and in those treated with enterocins A and B or commercial nisin combined with HHP. An increase of a_w was also detected in samples where enterocins A and B, pediocin PA-1 or nisin Z were applied individually. Along the storage period, values of a_w varied between 0.932 and 0.912 with a maximum difference of 0.02 units, which might not be considered important in terms of quality or safety of beef carpaccio. These results are in accordance with De Alba et al. (2012) who observed that the maximum difference of a_w between non-treated and pressurized carpaccio was 0.01 units. The cook loss of beef was higher at 300 and 400 MPa than at lower intensity pressures (McArdle et al., 2010), suggesting that water binding properties of meat was negatively affected by higher pressures. Also, liquid exudation as a consequence of the decrease of water holding capacity (WHC) of beef meat treated at pressures higher than 345 MPa was observed by Sánchez-Basurto et al. (2012). This fact was due to the exposition of hydrophobic sites of meat proteins probably by protein denaturation. However, the decrease of WHC during storage at 4 ºC was associated with a decrease of pH, correlated with microbial and enzyme activity, not being significantly affected by pressure. On the contrary, Liu et al. (2012) did not observe significant differences in a_w of sliced cooked ham treated at 400 MPa for 10 min in combination with enterocin LM-2, indicating that combined treatment of HHP and bacteriocin had no influence on a_w.

3.4. Color

The effect of treatments on color of vacuum-packaged cured beef carpaccio is shown in Table 7. Immediately after pressurization, *L** (lightness) slightly increased in carpaccio treated at 450 MPa for 5 min regarding control group. Bacteriocins applied individually did not affect *L** values. However, an increase of lightness was observed with all combined treatments, although this rising was significantly higher ($P<0.05$) only in samples treated with HHP in combination with pediocin PA-1 or commercial nisin. After 30 d at 8 ºC, *L** values decreased ($P<0.05$) with enterocins A and B and increased ($P<0.05$) in samples treated with commercial pediocin.

Values of *a** (redness) decreased ($P<0.05$) both in samples where HHP was applied individually or combined with bacteriocins, immediately after pressurization. Among bacteriocins, only the individual application of nisin Z decreased ($P<0.05$) *a** values at 0 h. However, redness tended to attenuate with all treatments at the end of storage.

After pressurization, *b** (yellowness) tended to decrease in samples where HHP was applied alone or combined with bacteriocins, although this decrease was significant ($P<0.05$) only when pediocin PA-1 or commercial pediocin were combined with HHP. After 30 d at 8 ºC, differences in values of *b** tended to attenuate.

According to Liu et al. (2012), the combination of HHP at 200 or 400 MPa for 10 min and enterocin LM-2 had little influence on *L** and *b** values measured in sliced cooked ham stored under refrigeration at 4 ºC during 90 d. However, one day after pressurization combined treatments of the bacteriocin with 200 MPa reduced *a** values compared with non-treated samples. Values of *a** tended to decrease in all treated samples with the time of storage. On the other hand, De Alba et al. (2013), observed that changes in color properties of dry-cured ham treated at 400 or 500 MPa for 10 min individually or in combination with commercial nisin or pediocin were low, and values of *L**, *a** and *b** tended to decrease during 60 d of storage at 8 ºC.

Due to the fact that colour of the food surface is the first quality parameter evaluated by consumers, and that is critical to product acceptance (Leon et al., 2006),

preservation technologies of foods should affect minimally to this characteristic. It has been shown that changes in colour of raw cured meat products treated by HHP are less severe than the reported in raw meat, probably due to the protective action of nitric oxide on myoglobin (Goutefongea et al., 1995; Andersen et al., 1990). In this sense, De Alba et al. (2012) observed a slight whitening and lower redness of raw cured beef carpaccio pressurized at 450 MPa for 5, 10 and 15 min. However, this effect was higher when the time of pressurization increased. The results obtained in the present work show that the changes detected in beef carpaccio were mainly due to the effect of HHP and time of storage instead of bacteriocins.

3.5. Texture

Textural properties of cured beef carpaccio stored during 30 d at 8 ºC are shown in Table 8. Immediately after pressurization, no differences regarding hardness (N) were detected between non-treated and treated carpaccio, except with commercial pediocin applied individually or in combination with HHP. After 30 d at 8 ºC, hardness only increased (P<0.05) in non-treated carpaccio and in samples treated with enterocins A and B or combined with HHP.

No significant differences were observed in shear strength values (N), immediately after pressurization. However, an increase (P<0.05) of this property was detected after 30 d of storage at 8 ºC in samples treated with enterocins A and B, pediocin PA-1, nisin Z, or commercial pediocin, and also in pressurized samples combined with nisin Z or commercial nisin.

The effect of lactocin 705 and lactocin AL705 producing *Lactobacillus curvatus* CRL705 on tissue microstructure of vacuum-packed raw beef during 60 d at 2 ºC was evaluated by Castellano et al. (2010), observing by light microscopy that the microstructural changes on beef steaks showed a 10 d delay in the appearance of tissue degradation signs, being attributed not only to the number but to the species of bacteria present on refrigerated vacuum-packed meat such as lactic acid bacteria, *Enterobacteriaceae* and *B. thermosphacta* (Ercolini et al., 2006; Fontana et al., 2006;

Sakala et al., 2002). Since the bacteriocins produced by *L. curvatus* CRL705 demonstrated to be bacteriostatic against *B. thermosphacta*, major microstructural changes in beef steaks were prevented. No differences in Warner-Bratzler force in beef steaks, previously cooked in an electric grill, cooled to <10 ºC and kept at 8 ºC during approximately one hour, were detected by the evaluation panel among treatments during storage at 2 ºC. A tendency to increase instrumental tenderness as occur during beef ageing was also observed in *L. curvatus* CRL705 inoculated samples, confirming that the inoculation with the bioprotective culture did not affect ageing development. De Alba et al. (2012) observed a decrease of shear strength (N) in pressurized beef carpaccio at 450 MPa for 5, 10 and 15 min compared to control samples, immediately after treatments, although differences were lower when the time of pressurization increased. On the other hand, no significant differences in maximum force (N) were detected after treatments between non-pressurized and HHP treated carpaccio. However, both texture parameters increased in treated samples during storage at 8 ºC. Hardness of sliced cooked ham one day after pressurization was affected by HHP at 400 MPa for 10 min combined with enterocin LM-2 (Liu et al., 2012) and sensory parameters such as hardness, juiciness and overall acceptability of the product decreased during storage under refrigeration at 4 ºC. De Alba et al. (2013) found that shear strength values (N) determined with Warner-Bratzler blade decreased in sliced dry-cured ham subjected at 500 MPa for 10 min or treated with commercial nisin or pediocin, applied individually, one day after treatments. Similar results were obtained for hardness, with higher (P<0.05) values in samples treated with nisin or with the combination of 400 MPa and nisin or pediocin compared with control dry-cured ham. However, both textural properties did not show a clear trend during refrigerated storage at 8 ºC.

HHP causes protein denaturation, aggregation or gelation, which can result in meat becoming either tenderized or toughened, depending on the meat protein system, the temperature, the pressure, and its duration (Sun and Holley, 2010).

According to our results, high-pressure treatment at 450 MPa for 5 min sensitized *E. coli* O157:H7 cells to enterocins A and B, pediocin PA-1 or commercial nisin, exhibiting a synergistic antimicrobial effect of approximately 2 log units higher than the sum of individual activities after 30 d of storage at 8 ºC. Bacteriocins applied individually only affect *L. monocytogenes* counts, being commercial pediocin the most effective bacteriocin against the pathogen. Pressurization increased *L** and decreased *a** and *b** values. No differences were detected in textural properties immediately after pressurization, although hardness tended to increase with most of treatments at the end of storage. Further studies on HHP combined with other bacteriocins to evaluate their effectiveness on the inactivation of foodborne pathogens inoculated in beef carpaccio and the effect of treatments on quality of this meat product should be carried out.

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Table 1. Levels (log cfu/g) of *L. monocytogenes* in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC.

	0 h	1 d	3 d	7 d	15 d	30 d
Control	6.67 ± 0.30 ^{gB}	6.31 ± 0.51 ^{eA}	6.31 ± 0.74 ^{fgA}	6.49 ± 0.31 ^{hAB}	6.51 ± 0.31 ^{fAB}	6.40 ± 0.50 ^{fAB}
HHP	5.58 ± 0.53 ^{dE}	5.23 ± 0.97 ^{cD}	5.00 ± 0.83 ^{cdC}	5.07 ± 0.72 ^{cdCD}	4.11 ± 0.16 ^{cA}	4.48 ± 1.20 ^{bB}
Enterocins A and B	6.47 ± 0.41 ^{fgC}	5.77 ± 1.02 ^{dA}	6.08 ± 0.70 ^{fB}	6.20 ± 0.60 ^{fgB}	5.78 ± 0.68 ^{eA}	6.34 ± 0.47 ^{fBC}
Pediocin PA-1	6.03 ± 0.94 ^{eC}	5.93 ± 0.85 ^{dBC}	5.71 ± 1.04 ^{eA}	5.93 ± 0.54 ^{fBC}	5.77 ± 0.56 ^{eAB}	6.33 ± 0.40 ^{fD}
Nisin Z	6.57 ± 0.38 ^{fgB}	6.43 ± 0.45 ^{eAB}	6.37 ± 0.57 ^{gA}	6.42 ± 0.51 ^{ghAB}	6.37 ± 0.50 ^{fA}	6.47 ± 0.61 ^{fAB}
Commercial Nisin	6.25 ± 0.50 ^{efE}	4.63 ± 0.23 ^{bA}	4.96 ± 0.36 ^{cdB}	5.40 ± 0.76 ^{eC}	4.59 ± 0.41 ^{dA}	5.66 ± 0.70 ^{dD}
Commercial Pediocin	5.18 ± 0.94 ^{bcB}	4.58 ± 0.45 ^{bA}	4.91 ± 0.49 ^{cAB}	4.82 ± 0.35 ^{bcAB}	4.55 ± 0.26 ^{dA}	5.93 ± 0.28 ^{eC}
HHP+Enterocins A and B	5.12 ± 0.62 ^{bcE}	4.60 ± 0.72 ^{bD}	4.06 ± 0.28 ^{bB}	4.74 ± 0.58 ^{bD}	3.69 ± 0.13 ^{bA}	4.25 ± 0.40 ^{bC}
HHP+Pediocin PA-1	5.05 ± 0.99 ^{bE}	4.49 ± 0.54 ^{bC}	3.98 ± 0.02 ^{bA}	5.11 ± 0.37 ^{deE}	4.21 ± 0.42 ^{cB}	4.76 ± 0.81 ^{cD}
HHP+Nisin Z	5.49 ± 0.12 ^{cdD}	5.35 ± 0.17 ^{cD}	5.15 ± 0.13 ^{dC}	5.35 ± 0.15 ^{deD}	4.73 ± 0.42 ^{dB}	4.29 ± 0.11 ^{bA}
HHP+Com. Nisin	4.90 ± 0.81 ^{bD}	4.55 ± 0.27 ^{bC}	4.00 ± 0.07 ^{bB}	4.69 ± 0.15 ^{bCD}	3.80 ± 0.41 ^{bAB}	3.59 ± 0.27 ^{aA}
HHP+Com. Pediocin	4.19 ± 0.37 ^{aC}	3.83 ± 0.22 ^{aB}	3.70 ± 0.37 ^{aB}	3.70 ± 0.19 ^{aB}	3.23 ± 0.14 ^{aA}	3.65 ± 0.15 ^{aB}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 2. Levels (log cfu/g) of *S.* Enteritidis in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC.

	0 h	1 d	3 d	7 d	15 d	30 d
Control	5.76 ± 0.53 ^{eC}	5.55 ± 0.22 ^{fBC}	5.77 ± 0.50 ^{gC}	5.50 ± 0.51 ^{eBC}	4.86 ± 0.15 ^{efA}	5.35 ± 0.67 ^{efB}
HHP	1.63 ± 0.04 ^{aE}	1.56 ± 0.08 ^{cD}	1.61 ± 1.86 ^{bE}	0.45 ± 0.52 ^{aC}	0.35 ± 0.40 ^{bcB}	0.24 ± 0.28 ^{aA}
Enterocins A and B	5.83 ± 0.28 ^{eC}	5.80 ± 0.05 ^{gC}	5.56 ± 0.28 ^{efB}	5.79 ± 0.08 ^{fC}	5.22 ± 0.23 ^{ghA}	5.81 ± 0.62 ^{hC}
Pediocin PA-1	5.93 ± 0.45 ^{efB}	5.99 ± 0.07 ^{gB}	5.61 ± 0.16 ^{fgA}	5.45 ± 0.14 ^{eA}	5.51 ± 0.30 ^{hiA}	5.58 ± 0.69 ^{gA}
Nisin Z	5.75 ± 0.30 ^{eBC}	5.93 ± 0.05 ^{gC}	5.58 ± 0.02 ^{efgBC}	5.59 ± 0.11 ^{efBC}	4.78 ± 0.44 ^{eA}	5.51 ± 1.31 ^{fgB}
Commercial Nisin	5.83 ± 0.02 ^{eB}	5.77 ± 0.12 ^{fgB}	5.62 ± 0.36 ^{fgB}	5.12 ± 0.24 ^{dA}	5.15 ± 0.51 ^{fgA}	5.26 ±1.63 ^{eA}
Commercial Pediocin	6.16 ± 0.07 ^{fD}	5.95 ± 0.06 ^{gCD}	5.41 ± 0.59 ^{eAB}	5.41 ± 0.29 ^{eAB}	5.69 ± 0.12 ^{iBC}	5.30 ± 1.68 ^{eA}
HHP+Enterocins A and B	3.24 ± 0.07 ^{dE}	2.10 ± 0.95 ^{dD}	2.11 ± 1.29 ^{cD}	1.80 ± 0.83 ^{cC}	1.05 ± 0.31 ^{dB}	0.64 ± 0.74 ^{bA}
HHP+Pediocin PA-1	3.01 ± 0.28 ^{dE}	1.72 ± 0.38 ^{cD}	0.57 ± 0.31 ^{aB}	0.30 ± 0.35 ^{aA}	0.60 ± 0.35 ^{cBC}	0.66 ± 0.76 ^{bC}
HHP+Nisin Z	2.21 ± 0.98 ^{cF}	1.30 ± 0.25 ^{bC}	1.50 ± 0.01 ^{bD}	1.66 ± 0.95 ^{bcE}	0.30 ± 0.00 ^{abcA}	1.07 ± 1.23 ^{cdB}
HHP+Com. Nisin	1.68 ± 1.60 ^{abD}	0.24 ± 0.28 ^{aB}	0.39 ± 0.45 ^{aB}	0.24 ± 0.28 ^{aB}	0.00 ± 0.00 ^{aA}	0.93 ± 1.08 ^{cC}
HHP+Com. Pediocin	1.98 ± 1.94 ^{bcD}	3.28 ± 0.66 ^{eE}	3.90 ± 0.48 ^{dF}	1.51 ± 0.11 ^{bC}	0.15 ± 0.17 ^{abA}	1.14 ± 1.32 ^{dB}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 3. Levels (cfu/g) of *Escherichia coli* O157:H7 in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC.

	0 h	1 d	3 d	7 d	15 d	30 d
Control	6.23 ± 0.48 ^{dA}	6.73 ± 0.08 ^{efBC}	6.84 ± 0.23 ^{dC}	6.62 ± 0.11 ^{efBC}	6.51 ± 0.29 ^{eAB}	6.24 ± 0.40 ^{eA}
HHP	3.51 ± 0.11 ^{abB}	4.26 ± 0.79 ^{dC}	3.13 ± 1.05 ^{bA}	4.06 ± 0.26 ^{cC}	4.16 ± 0.44 ^{dC}	3.69 ± 0.14 ^{dB}
Enterocins A and B	6.65 ± 0.43 ^{eAB}	6.78 ± 0.14 ^{efAB}	6.84 ± 0.17 ^{dB}	6.70 ± 0.18 ^{efAB}	6.63 ± 0.25 ^{eAB}	6.58 ± 0.26 ^{fA}
Pediocin PA-1	6.65 ± 0.38 ^{eABC}	6.76 ± 0.09 ^{efBC}	6.75 ± 0.18 ^{dABC}	6.83 ± 0.16 ^{fC}	6.56 ± 0.32 ^{eAB}	6.52 ± 0.41 ^{fA}
Nisin Z	6.70 ± 0.15 ^{eBC}	6.57 ± 0.16 ^{eBC}	6.65 ± 0.08 ^{dBC}	6.16 ± 0.21 ^{dA}	6.81 ± 0.11 ^{eC}	6.52 ± 0.15 ^{fB}
Commercial Nisin	6.61 ± 0.07 ^{eBC}	6.60 ± 0.22 ^{eBC}	6.70 ± 0.23 ^{dC}	6.46 ± 0.29 ^{eA}	6.54 ± 0.17 ^{eAB}	6.64 ± 0.05 ^{fBC}
Commercial Pediocin	6.62 ± 0.10 ^{eA}	6.95 ± 0.08 ^{fD}	6.80 ± 0.07 ^{dBC}	6.68 ± 0.12 ^{efAB}	6.69 ± 0.04 ^{eAB}	6.84 ± 0.10 ^{gCD}
HHP+Enterocins A and B	3.35 ± 0.14 ^{aB}	3.53 ± 0.66 ^{bBC}	3.64 ± 0.64 ^{cBC}	3.71 ± 0.51 ^{bC}	3.60 ± 1.28 ^{bBC}	1.73 ± 0.26 ^{aA}
HHP+Pediocin PA-1	3.74 ± 0.66 ^{bC}	2.97 ± 0.26 ^{aB}	3.32 ± 0.46 ^{bB}	3.30 ± 0.27 ^{aB}	3.06 ± 0.24 ^{aB}	1.74 ± 0.07 ^{aA}
HHP+Nisin Z	4.32 ± 0.11 ^{cE}	4.10 ± 0.40 ^{cdD}	3.70 ± 0.09 ^{cB}	3.83 ± 0.13 ^{bcBC}	3.97 ± 0.21 ^{cdCD}	3.21 ± 0.72 ^{cA}
HHP+Com. Nisin	3.64 ± 1.73 ^{abD}	3.41 ± 1.46 ^{bCD}	2.85 ± 0.32 ^{aB}	3.34 ± 0.21 ^{aC}	3.43 ± 0.56 ^{bCD}	2.17 ± 1.61 ^{bA}
HHP+Com. Pediocin	4.42 ± 0.97 ^{cE}	3.97 ± 0.57 ^{cD}	3.88 ± 0.39 ^{cCD}	3.57 ± 0.29 ^{abB}	3.69 ± 0.13 ^{bcBC}	3.12 ± 0.99 ^{cA}

Values are the mean ± SD.

Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.

Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 4. Levels (log cfu/g) of total viable counts (TVC) in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC.

	0 h	1 d	7 d	15 d	30 d
Control	7.09 ± 0.45 ^{eA}	7.08 ± 0.24 ^{efA}	7.84 ± 0.35 ^{dB}	7.99 ± 0.64 ^{cB}	8.03 ± 0.58 ^{defB}
HHP	4.95 ± 1.04 ^{bA}	4.83 ± 1.05 ^{bA}	4.92 ± 1.08 ^{aA}	5.73 ± 1.68 ^{abA}	7.01 ± 1.09 ^{bcB}
Enterocins A and B	6.73 ± 0.30 ^{eA}	6.69 ± 0.34 ^{deA}	8.28 ± 0.09 ^{dB}	8.57 ± 0.11 ^{cC}	8.59 ± 0.09 ^{fC}
Pediocin PA-1	6.54 ± 0.24 ^{deA}	6.81 ± 0.21 ^{deB}	8.10 ± 0.30 ^{dC}	8.39 ± 0.17 ^{cD}	8.44 ± 0.04 ^{efD}
Nisin Z	6.81 ± 0.47 ^{eB}	6.33 ± 0.17 ^{dA}	7.65 ± 0.49 ^{dC}	8.46 ± 0.08 ^{cD}	8.51 ± 0.07 ^{efD}
Commercial Nisin	5.91 ± 0.33 ^{cdA}	6.54 ± 0.44 ^{deB}	6.46 ± 0.21 ^{cB}	6.51 ± 0.36 ^{bB}	7.40 ± 0.23 ^{bcdC}
Commercial Pediocin	7.17 ± 0.63 ^{eA}	7.41 ± 0.48 ^{fA}	7.58 ± 0.12 ^{dA}	8.04 ± 0.48 ^{cB}	8.27 ± 0.21 ^{defB}
HHP+Enterocins A and B	5.74 ± 0.17 ^{cB}	5.58 ± 0.17 ^{cAB}	5.43 ± 0.13 ^{abA}	6.43 ± 0.62 ^{bC}	8.14 ± 0.53 ^{defD}
HHP+Pediocin PA-1	5.83 ± 0.22 ^{cdAB}	5.57 ± 0.19 ^{cA}	5.89 ± 0.04 ^{bcBC}	6.20 ± 0.35 ^{abC}	7.94 ± 0.55 ^{defD}
HHP+Nisin Z	5.57 ± 0.14 ^{bcA}	5.55 ± 0.19 ^{cA}	5.42 ± 0.21 ^{abA}	5.93 ± 0.48 ^{abB}	7.59 ± 0.60 ^{cdeC}
HHP+Com. Nisin	4.17 ± 0.95 ^{aA}	3.94 ± 1.12 ^{aA}	5.53 ± 0.41 ^{abC}	5.11 ± 0.70 ^{aB}	6.04 ± 0.36 ^{aD}
HHP+Com. Pediocin	4.15 ± 1.13 ^{aA}	4.48 ± 1.38 ^{abB}	6.03 ± 0.12 ^{bcC}	6.00 ± 0.18 ^{abC}	6.61 ± 1.06 ^{abD}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 5. Values of pH in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC.

	0 h	1 d	7 d	15 d	30 d
Control	5.44 ± 0.16 ^{cdC}	5.36 ± 0.17 ^{abBC}	5.16 ± 0.23 ^{abcAB}	5.05 ± 0.40 ^{abcA}	4.98 ± 0.25 ^{cdefA}
HHP	5.38 ± 0.10 ^{bcdB}	5.37 ± 0.12 ^{abB}	5.26 ± 0.21 ^{bcdB}	5.29 ± 0.38 ^{cB}	5.01 ± 0.18 ^{defA}
Enterocins A and B	5.37 ± 0.11 ^{bcdC}	5.39 ± 0.11 ^{abC}	4.94 ± 0.23 ^{aB}	4.95 ± 0.53 ^{abB}	4.84 ± 0.08 ^{abcdA}
Pediocin PA-1	5.25 ± 0.06 ^{aC}	5.39 ± 0.20 ^{abD}	4.96 ± 0.10 ^{aB}	4.76 ± 0.29 ^{aA}	4.73 ± 0.23 ^{aA}
Nisin Z	5.38 ± 0.15 ^{bcdC}	5.40 ± 0.15 ^{abC}	5.04 ± 0.20 ^{abB}	4.94 ± 0.43 ^{abA}	4.86 ± 0.14 ^{abcdeA}
Commercial Nisin	5.31 ± 0.17 ^{abC}	5.31 ± 0.14 ^{aC}	5.34 ± 0.08 ^{cdC}	5.15 ± 0.07 ^{bcB}	5.04 ± 0.16 ^{fA}
Commercial Pediocin	5.37 ± 0.13 ^{bcdD}	5.32 ± 0.12 ^{aC}	5.29 ± 0.09 ^{cdC}	5.05 ± 0.05 ^{abcB}	4.82 ± 0.12 ^{abcA}
HHP+Enterocins A and B	5.45 ± 0.14 ^{cdC}	5.43 ± 0.24 ^{abC}	5.29 ± 0.39 ^{cdB}	5.07 ± 0.25 ^{abcA}	5.04 ± 0.06 ^{fA}
HHP+Pediocin PA-1	5.37 ± 0.10 ^{bcdCD}	5.39 ± 0.12 ^{abD}	5.30 ± 0.34 ^{cdC}	4.92 ± 0.38 ^{abB}	4.79 ± 0.14 ^{abA}
HHP+Nisin Z	5.47 ± 0.08 ^{dC}	5.46 ± 0.07 ^{bC}	5.35 ± 0.18 ^{cdB}	5.10 ± 0.10 ^{bcA}	5.02 ± 0.36 ^{efA}
HHP+Com. Nisin	5.33 ± 0.16 ^{abB}	5.32 ± 0.17 ^{abB}	5.40 ± 0.10 ^{dB}	5.34 ± 0.12 ^{cB}	5.24 ± 0.37 ^{gA}
HHP+Com. Pediocin	5.36 ± 0.10 ^{bcBC}	5.40 ± 0.10 ^{abC}	5.39 ± 0.11 ^{dBC}	5.32 ± 0.13 ^{cB}	4.96 ± 0.32 ^{bcdefA}

Values are the mean ± SD.
Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.
Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 6. Values of a_w in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC.

	0 h	1 d	7 d	15 d	30 d
Control	0.922 ± 0.006 ^{abBC}	0.926 ± 0.003 ^{abC}	0.920 ± 0.006 ^{bAB}	0.916 ± 0.007 ^{aA}	0.918 ± 0.004 ^{aA}
HHP	0.927 ± 0.004 ^{cdeA}	0.928 ± 0.004 ^{bA}	0.925 ± 0.004 ^{cdeA}	0.927 ± 0.005 ^{cdefA}	0.926 ± 0.005 ^{deA}
Enterocins A and B	0.931 ± 0.004 ^{eD}	0.929 ± 0.003 ^{bcBC}	0.930 ± 0.002 ^{efCD}	0.928 ± 0.002 ^{defB}	0.924 ± 0.003 ^{cdA}
Pediocin PA-1	0.930 ± 0.003 ^{eD}	0.927 ± 0.007 ^{abBC}	0.928 ± 0.003 ^{cdefC}	0.925 ± 0.003 ^{bcdeB}	0.921 ± 0.004 ^{bcA}
Nisin Z	0.929 ± 0.003 ^{deB}	0.927 ± 0.001 ^{abB}	0.928 ± 0.005 ^{defB}	0.924 ± 0.002 ^{bcdA}	0.924 ± 0.003 ^{cdA}
Commercial Nisin	0.922 ± 0.004 ^{abA}	0.928 ± 0.003 ^{bC}	0.924 ± 0.004 ^{bcdB}	0.922 ± 0.004 ^{bcA}	0.921 ± 0.005 ^{bcA}
Commercial Pediocin	0.920 ± 0.005 ^{aD}	0.925 ± 0.009 ^{aE}	0.912 ± 0.013 ^{aA}	0.915 ± 0.010 ^{aB}	0.918 ± 0.004 ^{abC}
HHP+Enterocins A and B	0.930 ± 0.003 ^{eB}	0.929 ± 0.004 ^{bcAB}	0.928 ± 0.003 ^{defA}	0.930 ± 0.003 ^{efAB}	0.928 ± 0.003 ^{eAB}
HHP+Pediocin PA-1	0.924 ± 0.005 ^{abcA}	0.928 ± 0.003 ^{bB}	0.931 ± 0.004 ^{fC}	0.931 ± 0.004 ^{fC}	0.928 ± 0.001 ^{eB}
HHP+Nisin Z	0.925 ± 0.002 ^{bcdA}	0.928 ± 0.005 ^{abB}	0.930 ± 0.003 ^{fC}	0.932 ± 0.003 ^{fC}	0.926 ± 0.001 ^{deA}
HHP+Com. Nisin	0.931 ± 0.002 ^{eB}	0.931 ± 0.005 ^{cB}	0.929 ± 0.005 ^{efA}	0.931 ± 0.003 ^{fB}	0.932 ± 0.001 ^{fB}
HHP+Com. Pediocin	0.923 ± 0.004 ^{abcB}	0.927 ± 0.004 ^{abC}	0.923 ± 0.005 ^{bcB}	0.921 ± 0.009 ^{bA}	0.923 ± 0.003 ^{cAB}

Values are the mean ± SD

Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.

Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 7. Color parameters in cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC.

		0 h	7 d	15 d	30 d
L*	Control	32.82±2.97^{abcdA}	33.64±2.67^{abcdA}	33.67±2.65^{abcdA}	32.59±2.50^{bcA}
	HHP	35.08±3.66^{defAB}	35.84±3.01^{eB}	35.55±2.42^{dB}	33.75±2.19^{bcdA}
	Ent. A-B	32.38±3.47^{abB}	33.18±2.34^{aB}	32.68±1.78^{abB}	30.17±2.50^{aA}
	Ped. PA-1	32.62±3.03^{abcA}	33.26±1.34^{abA}	31.82±1.95^{aA}	31.75±2.84^{abA}
	Nisin Z	32.00±2.79^{aA}	33.88±3.45^{abcdB}	31.90±2.11^{aA}	31.67±2.10^{abA}
	Com. Nisin	33.30±2.63^{abcdeA}	34.44±0.98^{abcdeB}	32.38±1.74^{aA}	33.46±2.22^{bcdAB}
	Com. Pediocin	32.89±2.63^{abcdA}	33.48±2.68^{abcA}	33.39±3.26^{abcA}	35.47±3.83^{deB}
	HHP+Ent A-B	34.48±3.39^{bcdefA}	35.37±2.38^{cdeA}	35.07±2.12^{cdA}	35.09±2.93^{deA}
	HHP+Ped. PA-1	35.20±2.82^{efA}	35.48±2.44^{deA}	35.46±2.51^{dA}	34.29±2.64^{cdeA}
	HHP+Nisin Z	34.79±2.94^{cdefAB}	35.12±2.58^{bcdeB}	34.50±1.93^{bcdAB}	33.62±1.71^{bcdA}
	HHP+Com.Nis.	35.83±1.96^{fA}	35.96±2.00^{eA}	35.30±1.47^{dA}	35.98±2.35^{eA}
	HHP+Com.Ped.	34.89±2.00^{cdefA}	36.30±1.58^{eB}	34.62±2.24^{cdA}	35.38±2.03^{deAB}
*a**	Control	22.33±2.82^{fB}	21.13±3.77^{defB}	19.23±3.66^{aA}	22.66±3.79^{deB}
	HHP	17.82±2.66^{abA}	17.27±3.88^{abA}	20.20±3.65^{aB}	19.65±3.70^{abcB}
	Ent. A-B	23.48±2.82^{fB}	19.40±6.06^{bcdA}	20.29±3.11^{aA}	23.07±4.69^{eB}
	Ped. PA-1	22.93±3.20^{fB}	21.99±3.84^{efAB}	20.28±1.96^{aA}	20.38±2.02^{abcdA}
	Nisin Z	20.01±1.95^{cdA}	20.04±6.15^{cdeA}	20.22±3.87^{aA}	20.12±4.44^{abcA}
	Com. Nisin	22.22±3.07^{fA}	22.65±2.31^{fA}	21.26±2.99^{aA}	21.70±3.21^{bcdeA}
	Com. Pediocin	21.94±3.07^{efA}	20.79±4.62^{defA}	23.86±2.34^{bB}	21.89±4.78^{cdeA}
	HHP+Ent AB	18.31±2.59^{abcA}	19.21±2.65^{bcdAB}	20.71±2.84^{aB}	18.94±3.69^{aAB}
	HHP+Ped. PA-1	16.67±1.76^{aA}	16.64±3.70^{aA}	19.84±2.43^{aB}	20.26±2.72^{abcB}
	HHP+Nisin Z	18.62±2.99^{bcdAB}	17.76±2.56^{abcA}	19.97±2.96^{aB}	19.66±2.32^{abcB}
	HHP+Com.Nis.	20.30±2.52^{deB}	18.70±2.17^{abcdA}	20.91±1.97^{aB}	20.46±3.02^{abcdB}
	HHP+Com.Ped.	19.75±2.18^{cdA}	18.76±2.00^{abcdA}	18.82±3.18^{aA}	19.57±3.13^{abA}
*b**	Control	12.78±5.31^{bcdB}	9.80±2.45^{cdeA}	8.16±2.96^{aA}	12.06±5.78^{efB}
	HHP	10.48±3.46^{abB}	8.52±2.06^{abcA}	10.01±2.95^{abcAB}	9.81±4.08^{abcdAB}
	Ent. A-B	13.95±3.98^{dB}	9.75±2.53^{cdeA}	8.51±3.16^{abA}	13.09±6.98^{fB}
	Ped. PA-1	13.47±4.23^{cdC}	10.62±1.30^{eB}	8.39±2.64^{abA}	11.07±5.18^{bcdefB}
	Nisin Z	10.33±2.31^{abBC}	10.09±3.97^{deB}	8.52±4.20^{abA}	11.59±6.58^{cdefC}
	Com. Nisin	12.72±6.77^{bcdC}	10.14±4.13 ^{deB}	8.58±2.42^{abA}	9.04±2.63^{abAB}
	Com. Pediocin	12.28±6.46^{abcdB}	9.45±4.36^{bcdeA}	11.24±2.50^{cAB}	10.78±4.74^{abcdeAB}
	HHP+Ent AB	10.63±3.49^{abAB}	9.29±1.40^{abcdeA}	10.20±3.71^{bcAB}	11.11±5.56^{bcdefB}
	HHP+Ped. PA-1	9.92±1.75^{aB}	8.53±1.39^{abcA}	9.24±3.09^{abcAB}	11.69±5.03^{defC}
	HHP+Nisin Z	11.12±4.00^{abcB}	8.89±1.26^{abcdA}	8.93±3.21^{abA}	11.50±4.86^{cdefB}
	HHP+Com.Nis.	11.05±5.32^{abcC}	7.93±2.39^{aA}	9.12±2.42^{abAB}	9.46±2.39^{abcB}
	HHP+Com.Ped.	10.14±4.55^{aB}	8.24±2.79^{abA}	8.48±1.82^{abA}	8.60±2.02^{aA}

Values are the mean ± SD.

Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.

Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Table 8. Texture properties of cured beef carpaccio treated with HHP at 450 MPa, bacteriocins and their combinations, during 30 d at 8 ºC.

		0 h	7 d	15 d	30 d
Hardness (N)	Control	107.6 ± 34.0 ^{abA}	114.0 ± 45.5 ^{abcAB}	131.6 ± 43.9 ^{bcdAB}	139.9 ± 48.7 ^{abcB}
	HHP	132.5 ± 46.4 ^{bcA}	140.9 ± 60.4 ^{cdA}	129.8 ± 31.6 ^{bcdA}	149.6 ± 37.8 ^{bcA}
	Enterocins A-B	82.0. ± 31.5 ^{aA}	121.7 ± 46.8 ^{abcdB}	120.7 ± 25.7 ^{abcdB}	119.2 ± 29.3 ^{abB}
	Pediocin PA-1	78.5 ± 30.7 ^{aA}	83.9 ± 24.5 ^{aA}	93.6 ± 22.3 ^{abA}	103.3 ± 33.3 ^{aA}
	Nisin Z	132.6 ± 36.1 ^{bcBC}	156.5 ± 47.2 ^{dC}	106.8 ± 27.3 ^{abA}	127.4 ± 34.4 ^{abAB}
	Com. Nisin	138.7 ± 41.8 ^{bcA}	130.9 ± 33.4 ^{bcdA}	149.4 ± 47.3 ^{cdA}	154.3 ± 31.1 ^{bcA}
	Com. Pediocin	154.9 ± 55.5 ^{cB}	91.2 ± 18.3 ^{abA}	156.5 ± 72.7 ^{dB}	179.3 ± 62.8 ^{cB}
	HHP + Ent. A-B	104.1 ± 47.7 ^{abA}	143.8 ± 37.1 ^{cdB}	82.9 ± 24.5 ^{aA}	133.9 ± 45.2 ^{abB}
	HHP + Pediocin PA-1	109.7 ± 25.3 ^{abA}	139.7 ± 48.2 ^{cdA}	131.7 ± 43.8 ^{bcdA}	117.4 ± 39.6 ^{abA}
	HHP + Nisin Z	100.8 ± 28.2 ^{abA}	152.81 ± 35.22 ^{cdB}	93.8 ± 16.4 ^{abA}	105.6 ± 30.2 ^{aA}
	HHP + Com. Nisin	118.9 ± 43.4 ^{abcA}	123.9 ± 36.3 ^{abcdA}	126.1 ± 36.4 ^{bcdA}	142.4 ± 45.2 ^{abcA}
	HHP + Com. Pediocin	158.0 ± 32.1 ^{cB}	114.1 ± 16.9 ^{abcA}	109.8 ± 30.1 ^{abcA}	150.0 ± 33.4 ^{bcB}
Shear force (N)	Control	33.4 ± 16.1 ^{abA}	31.7 ± 9.3 ^{abA}	29.2 ± 7.3 ^{aA}	34.9 ± 9.8 ^{aA}
	HHP	29.1 ± 9.9 ^{aA}	34.1 ± 13.0 ^{bBC}	38.7 ± 8.6 ^{bcdC}	32.1 ± 7.8 ^{aAB}
	Enterocins A-B	28.4 ± 8.6 ^{aA}	35.9 ± 14.2 ^{bB}	38.7 ± 10.2 ^{bcdB}	36.7 ± 9.2 ^{aB}
	Pediocin PA-1	33.2 ± 8.9 ^{abAB}	28.8 ± 11.7 ^{abA}	39.2 ± 14.7 ^{bcdB}	50.0 ± 18.8^{bC}
	Nisin Z	30.0 ± 7.3 ^{aA}	30.6 ± 10.7 ^{abA}	35.2 ± 6.9 ^{abcdAB}	39.3 ± 12.9 ^{aB}
	Com. Nisin	29.9 ± 12.9 ^{aA}	30.4 ± 10.9 ^{abA}	39.9 ± 9.5 ^{cdB}	33.6 ± 11.5 ^{aAB}
	Com. Pediocin	28.2 ± 9.1 ^{aA}	33.7 ± 14.2 ^{bAB}	32.6 ± 10.6 ^{abAB}	36.5 ± 7.8 ^{aB}
	HHP + Ent. A-B	34.9 ± 12.4 ^{abA}	31.9 ± 6.7 ^{abA}	34.8 ± 10.4 ^{abcdA}	36.7 ± 6.7 ^{aA}
	HHP + Pediocin PA-1	41.0 ± 10.7 ^{bB}	33.2 ± 8.3 ^{bA}	32.3 ± 9.7 ^{abA}	39.2 ± 12.5 ^{aAB}
	HHP + Nisin Z	27.6 ± 8.3 ^{aA}	25.5 ± 7.0 ^{aA}	41.2 ± 16.6 ^{dB}	46.7 ± 15.8 ^{bC}
	HHP + Com. Nisin	31.1 ± 6.0 ^{aA}	31.8 ± 7.1 ^{abAB}	36.3 ± 13.0 ^{abcdBC}	37.8 ± 8.8 ^{aC}
	HHP + Com. Pediocin	33.8 ± 8.1 ^{abA}	34.6 ± 8.2 ^{bA}	33.0 ± 7.5 ^{abcA}	32.8 ± 8.9 ^{aA}

Values are the mean ± SD.

Means within the same column with different lower-case superscripts differ significantly at $P<0.05$.

Means within the same row with different upper-case superscripts differ significantly at $P<0.05$.

Highlights

- Bacteriocins did not affect the survival of *S*. Enteritidis or *E*. *coli* O157-H7, although were effective on *L. monocytogenes* counts in beef carpaccio.

- A synergistic antimicrobial effect of HHP combined with enterocins A and B, pediocin PA-1 or commercial nisin was detected on *E. coli* O157-H7 in beef carpaccio.

- In general, color and texture were slightly affected by treatments.

3.8. Combined effect of sodium nitrite with high-pressure treatments on the inactivation of *Escherichia coli* BW25113 y *Listeria monocytogenes* NCTC 11994. *Letters in Applied Microbiology (2013) 56: 155-160.*

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ORIGINAL ARTICLE

Combined effect of sodium nitrite with high-pressure treatments on the inactivation of *Escherichia coli* BW25113 and *Listeria monocytogenes* NCTC 11994

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Significance and Impact of the Study: High hydrostatic pressure (HHP) is an effective nonthermal food preservation technology that can be used to increase food safety and shelf life with minimal changes in food quality. Various antimicrobial agents including bacteriocins, lysozyme, chitosan, lactoperoxidase and essential oils have been tested in combination with HHP to enhance the effect of mild-pressure treatments. This is the first report of a synergistic bactericidal effect of high pressure and acidified nitrite. A better understanding of combined preservation processes and synergistic effects will help ensure the safety of processed foods.

Keywords
Escherichia coli, high pressure, *hmp*A, *Listeria monocytogenes*, *nor*V, sodium nitrite.

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Abstract

The bactericidal effect of acidified sodium nitrite alone or when combined with high hydrostatic pressure (HHP) treatment was examined with *Escherichia coli* BW25113 and *Listeria monocytogenes* NCTC 11994. A powerful synergistic effect of HHP plus nitrite was observed at pH 4·0, but not at higher pH values. *Escherichia coli hmp*A and *nor*V mutants lacking defences against nitrosative stress were more sensitive to pressure combined with acidified sodium nitrite than the wild-type strain, suggesting an involvement of nitric oxide in the bactericidal effect.

Introduction

Nitrites and nitrates are used as food additives in processed foods, including cured and other processed meats, fish, poultry and some cheeses. Their use, usually as potassium or sodium salts, is limited in most countries. Sodium nitrite, known as E250, is used to stabilize the colour of preserved fish and meats and to inhibit the growth of *Clostridium botulinum*. Non-spore-forming spoilage organisms and pathogens are also inhibited by nitrite (Honikel 2008; EFSA 2009).

The bacteriostatic action of nitrite increases with decreasing pH, pointing to nitrous acid as the main inhibitor of microbial growth (Tompkin *et al.* 1978). Nitrous acid breaks down spontaneously to give nitric oxide ($NO^{\cdot}$), a free radical that can react with reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^{-}) to form a variety of antimicrobial molecular species that are more potent than nitric oxide itself. These include peroxynitrite ($^{-}OONO$), *S*-nitrosothiols (RSNO), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4) (Brunelli *et al.* 1995). Nitric oxide or its reaction products can inactivate iron-sulphur proteins such as clostridial ferredoxins or aerobic respiratory chain enzymes and can also react with protein thiols and cause deamination and other changes to DNA (Inoue and Kawanishi 1995; Juedes and Wogan 1996; Rahman 2007). Nitric oxide is involved in many physiological functions in the mammalian body and plays a major role in the bactericidal activity of

macrophages and neutrophils. Production of nitric oxide in the stomach from salivary nitrite has been suggested as an important host defence against foodborne pathogens (Dykhuizen *et al.* 1996).

The stability of pasteurized cured meat and fish products depends on the combined effect of nitrite with many other preservative factors, but the effect of heat is especially important in causing injury to spores that prevents their outgrowth. With regard to vegetative cells, there is little information on the possible effects of nitrite combined with physical stress treatments. High hydrostatic pressure (HHP) is an effective technology that can be used to increase food safety and shelf life with minimal changes in the organoleptic properties (San Martín *et al.* 2002; Considine *et al.* 2008). Combining nonthermal processes with conventional preservation methods can enhance their antimicrobial effect, allowing the use of lower pressurization intensities and additive concentrations. The mechanisms of inactivation of bacteria by HHP are not fully understood, although damage to membranes, denaturation of proteins and the generation of oxidative stress have been reported (Mackey and Mañas 2008). With regard to oxidative stress, there is evidence that pressure may lead to the generation of ROS within the cell (Aertsen *et al.* 2005). This suggests that HHP might enhance the antimicrobial effect of nitrite.

Listeria monocytogenes is a foodborne pathogen (McLauchlin *et al.* 2004) that causes listeriosis, a severe foodborne disease of particular concern for pregnant women, newborns and adults with a weakened immune system. Due to its psychrotrophic character, it is a pathogen of concern in refrigerated food products. *Listeria monocytogenes* can grow over a wide range of temperatures and pH values and tolerate high levels of sodium chloride and sodium nitrite (Rocourt *et al.* 2000). *Escherichia coli* is a normal inhabitant of the large intestine, but certain strains can cause serious foodborne illness and other infections (Pitout 2012). Some strains of *E. coli* are relatively resistant to nitrite (Gibson and Roberts 1986). The aim of this work was to investigate the bactericidal effect of sodium nitrite alone or in combination with HHP as a function of the medium pH. Studies were performed using *E. coli* BW25113 and *L. monocytogenes* NCTC 11994 representing Gram-negative and Gram-positive bacterial species and two *E. coli* BW25113 mutants lacking components of the nitrosative stress response.

Results and discussion

Bactericidal effect of acidified nitrite

Native cells of *E. coli* and *L. monocytogenes* were sensitive to sodium nitrite at pH 4·0. Exposure of *E. coli* to 1·0, 2·0 or 3·0 mmol l^{-1} acidified sodium nitrite caused an immediate reduction in viable counts of <1 log but after storage at 4°C for 24 h the log reductions increased to 1·1, 2·6 and 5 logs, respectively (Fig. 1). Regarding *L. monocytogenes* (Fig. 2), the presence of 0·25, 0·50 or 0·75 mmol l^{-1} sodium nitrite caused immediate reductions of up to 0·6 log increasing to 0·90, 2·3 and 3·6 logs, respectively, after 24 h. Analysis of variance revealed a significant influence of treatment and time of refrigeration ($P < 0·001$) on both *E. coli* and *L. monocytogenes*.

Most investigations into the effect of nitrite on *E. coli* and *L. monocytogenes* have sought to define the concentrations needed to inhibit growth in food or broth systems. The minimum inhibitory concentration of nitrite for *E. coli* and *L. monocytogenes* is highly dependent on pH, salt concentration and incubation temperature; hence, inhibitory levels may vary between about 100 and 400 ppm (1·4–5·8 mmol l^{-1}) depending on conditions (Gibson and Roberts 1986; McClure *et al.* 1991). The growth inhibitory action of nitrite on several species of bacteria increased at acidic pH when tested in nutrient broth and fish digest broth (Tarr 1941). At pH values below 4·5–5·0, the effect of nitrite becomes bactericidal for *E. coli* O157 (Buchanan and Bagi 1994; Casey and Condon 2000). Dykhuizen *et al.* (1996) noted a linear relationship between the logarithm of the minimum bactericidal concentration and pH values between 2·1 and 4·8 for several species of Gram-negative bacteria.

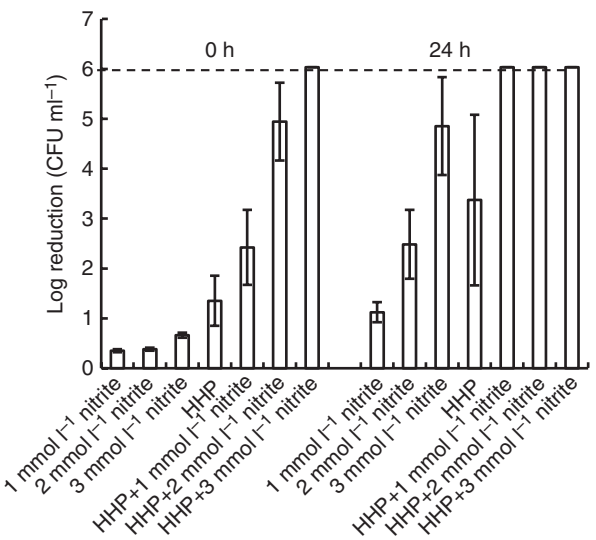

Figure 1 Effect of high hydrostatic pressure and nitrite separately or in combination with *Escherichia coli*. Log_{10} cycles of inactivation at pH 4·0 immediately after treatment (0 h) and after 24 h storage at 4°C. Pressure treatment was for 8 min at 300 MPa. Results are means of three observations ± standard deviations (error bars). Dashed line indicates maximum detectable log reduction.

Microbial inactivation by sodium nitrite combined with high-pressure treatment

With *E. coli*, a pressure treatment at 300 MPa for 8 min caused an average reduction in viable count of 1·4 logs (Fig. 1). When this pressure treatment was combined with 1·0 or 2·0 mmol l^{-1} sodium nitrite, viable numbers decreased by 2·4 and 4·9 logs, respectively, whilst with 3·0 mmol l^{-1} nitrite viable counts were below the limit of detection (<2·4 log CFU ml^{-1}) indicating reductions of >6 logs (Fig. 1). The degree of inactivation 24 h later was >6 logs in all the combined treatments. With *L. monocytogenes*, pressure treatment for 8 min at 225 MPa caused about a 1 log reduction in viability. When combined with 0·25, 0·50 or 0·75 mmol l^{-1} sodium nitrite, decreases of 1·5, 2·7 and 4·2 logs, respectively, were obtained (Fig. 2). An increase ($P < 0·001$) in inactivation was observed 24 h later achieving reductions of 3·4, 6·1 and 6·1 logs, respectively.

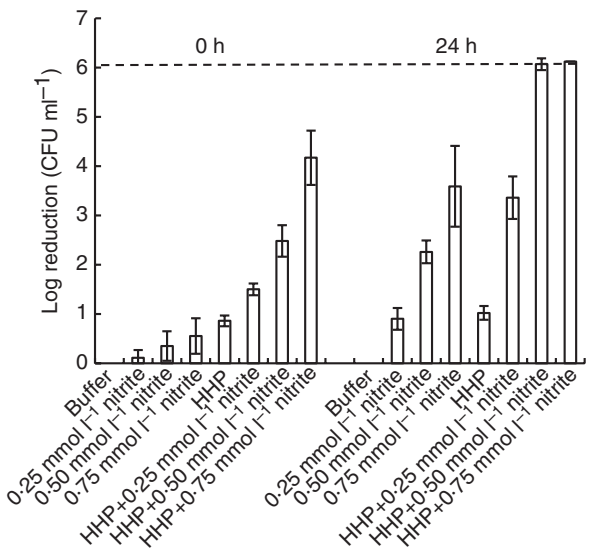

Figure 2 Effect of high hydrostatic pressure and nitrite separately or in combination with *Listeria monocytogenes*. Log_{10} cycles of inactivation at pH 4·0 immediately after treatment (0 h) and after 24 h storage at 4°C. Pressure treatment was for 8 min at 225 MPa. Results are means of three observations ± standard deviations (error bars). Dashed line indicates maximum detectable log reduction.

Synergistic effects

The extent of inactivation achieved by nitrite combined with HHP was considerably greater than the sum of the individual treatments applied separately (Table 1). With *E. coli*, up to 4 logs additional kill was seen within the first hour after exposure but this declined somewhat after further storage. With *L. monocytogenes*, up to 3 logs additional inactivation was seen. With 0·75 mmol l^{-1} nitrite, the synergistic effect declined after 24 h but increased with storage time at the two lower concentrations. The decrease in the synergistic effect with *E. coli* during storage was because viable numbers declined in pressure-treated cells remaining in the acid buffer and in non-pressure-treated cells in acidified nitrite. Nevertheless, the decline in viability under these conditions was less than with the combined treatment. The loss of viability of pressure-injured *E. coli* and *L. monocytogenes* following holding in acidic buffers or fruit juice is well known (García-Graells *et al.* 1998; Jordan *et al.* 2001). Jofré *et al.* (2010) recently reported that *Staphylococcus aureus* cells damaged by HHP treatment subsequently died when inoculated into media containing salt or nitrite, but there are no previous reports of a synergistic bactericidal effect of pressure combined with acidified nitrite.

Effect of pH on the inactivation of *Escherichia coli* BW25113 by HHP combined with nitrite

The effect of pH on high pressure combined with 2 mmol l^{-1} nitrite is shown in Fig. 3. Treatment at pH 4·0 caused reductions of about 4 logs immediately after treatment, but no effect was seen at pH 5·0 or pH 7·0. A slight synergistic effect was observed at pH 4·5 with a higher nitrite level of 3 mmol l^{-1}. Under these

Table 1 Synergistic effect* of acidified nitrite combined with high hydrostatic pressure (HHP)†

Time after HHP (h)	*Escherichia coli*			*Listeria monocytogenes*		
	1 mmol l^{-1} $NaNO_2$	2 mmol l^{-1} $NaNO_2$	3 mmol l^{-1} $NaNO_2$	0·25 mmol l^{-1} $NaNO_2$	0·5 mmol l^{-1} $NaNO_2$	0·75 mmol l^{-1} $NaNO_2$
0	0·72 ± 0·61^a	3·20 ± 1·07^b	4·03 ± 0·52^c	0·54 ± 0·33^a	1·27 ± 0·31^a	2·76 ± 0·31^b
1	2·09 ± 1·55^c	4·03 ± 0·72^c	3·75 ± 0·55^b	0·60 ± 0·47^a	1·70 ± 0·69^a	3·01 ± 0·29^b
24	1·59 ± 1·65^b	1·01 ± 1·49^a	0·26 ± 0·41^a	1·44 ± 0·45^b	2·79 ± 0·36^b	1·51 ± 0·92^a

*Log reductions (±standard deviation) achieved by the combined treatment minus the sum of log reductions achieved by individual nitrite and pressure treatments. In some individual experiments with *E. coli*, the calculated sum of the individual log reductions after 24 h exceeded the number of log reductions that could actually be measured. In these cases, the synergistic effect, if any, is unknown and was recorded as zero in calculating mean values. Means within the same column with different superscripts differ significantly at $P < 0·05$.
†Cells were pressure-treated for 8 min at 300 or 225 MPa for *E. coli* and *L. monocytogenes*, respectively.

conditions, the combined treatment caused a reduction of 1·4 log compared with 0·23 log for the sum of separate treatments (data not shown). The pH dependence is consistent with nitric oxide derived from nitrous acid being the active bactericidal agent.

Effect of sodium nitrite and high-pressure treatment on *Escherichia coli* BW25113 wild type, *hmp*A and *nor*V mutants

The *E. coli hmp*A and *nor*V mutants lacking the protective enzymes nitric oxide dioxygenase and nitric oxide reductase, respectively (Gardner *et al.* 2002; Poole 2005), were significantly ($P < 0·05$) more sensitive to acidified nitrite than the parent strain (Table 2). Unexpectedly, they were even more sensitive to HHP treatment alone. From Table 2, it can be calculated that the synergistic effects (the average increase in log reduction achieved by the combined treatment compared with the sum of individual treatments) for parent, *hmpA* and *norV* strains were 1·68, 2·01 and 2·33 logs, respectively. However, these differences in synergistic effect between strains were not statistically significant.

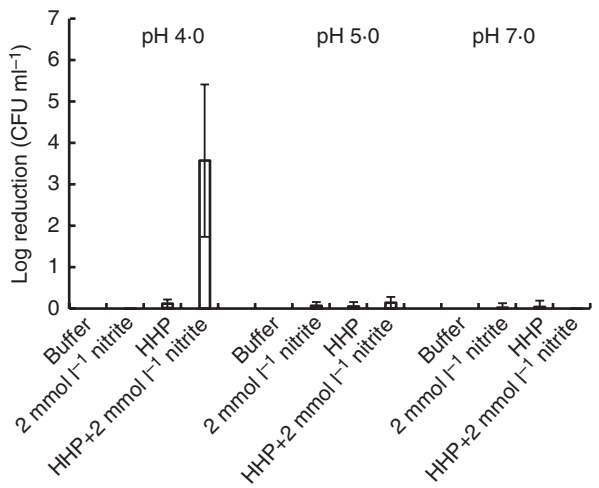

Figure 3 Effect of pH on inactivation of *Escherichia coli* by high hydrostatic pressure combined with nitrite. Pressure treatment was for 8 min at 300 MPa. Results are means of three observations ± standard deviations (error bars) determined after treatments.

The mechanism of the synergistic effect is not known but could be due to inactivation of protective systems against nitrosative stress or to the enhanced formation of reactive products such as peroxynitrite as a result of the generation of intracellular ROS during pressure treatment. A quite different effect of nitric oxide on pressure resistance was reported by Domitrovic *et al.* (2003) who found that low levels of nitric oxide delivered from donor compounds actually protected *Saccharomyces cerevisiae* against HHP. This was attributed to the triggering of a stress response by nitric oxide. The basis of the increase in pressure sensitivity in the *E. coli hmpA* and *norV* mutants even in the absence of acidified nitrite is not known. One possibility is that HHP might disrupt cellular metabolic processes resulting in increased internal NO production.

The combined use of antimicrobials (bacteriocins, lysozyme, chitosan, lactoperoxidase system, essential oils) together with HHP has been tested in searching for synergistic effects (Karatzas *et al.* 2001; Garriga *et al.* 2002; Somolinos *et al.* 2008). It would be useful to test possible synergistic effects of pressure plus nitrite in foods such as fermented meats and some cheeses in which nitrite is a permitted additive. Relatively low concentrations of nitrite (17–34 ppm) were sufficient to increase the bactericidal effect of HHP on *L. monocytogenes*, and further work should determine the effect of food components and process variables on the cell killing effect. Nitric oxide is an important molecule involved in cell signalling and host defences against infection as well as in food preservation. The effect of other physical stress treatment on microbial resistance to nitric oxide merits investigation.

Materials and methods

Bacterial strains and growth media

Escherichia coli BW25113 wild type and *E. coli* BW25113 *hmp*A and *nor*V mutants lacking the flavohaemoglobin nitric oxide dioxygenase and nitric oxide reductase,

Table 2 Effect of mutations affecting nitrosative stress resistance on inactivation of *Escherichia coli* by acidified nitrite alone or in combination with high hydrostatic pressure*

	pH 4·0	3 mmol l^{-1} $NaNO_2$	300 MPa/8 min	Combined treatment
Wild type	$0·00 \pm 0·01^{aA}$	$0·11 \pm 0·08^{aA}$	$0·93 \pm 0·19^{aB}$	$2·72 \pm 0·31^{aC}$
*hmp*A	$-0·02 \pm 0·03^{aA}$	$0·60 \pm 0·23^{bA}$	$3·22 \pm 0·72^{bB}$	$5·83 \pm 1·15^{bC}$
*nor*V	$0·04 \pm 0·02^{bA}$	$0·64 \pm 0·13^{bA}$	$3·52 \pm 0·46^{cB}$	$6·49 \pm 0·87^{cC}$

Numbers are log reductions in viable count (mean ± SD of duplicate determinations in three experiments). Means within the same column with different lower-case superscripts differ significantly at $P < 0·05$. Means within the same row with different upper-case superscripts differ significantly at $P < 0·05$.

*Cells were pressure-treated for 8 min at 300 MPa.

respectively, were from the Keio collection (Baba *et al.* 2006). These strains were kindly provided by Professor H. Mori, Keio University, Japan. *Listeria monocytogenes* NCTC 11994 was also used in this study. The strains were maintained frozen at −70°C on Cryobeads (Prolab Diagnostics, Neston, UK). Bacterial broth subcultures were prepared by inoculating 10 ml of tryptone soya broth (TSB CM129; Oxoid, Basingstoke, UK) with a single colony from a plate. The cultures were incubated at 37°C for 6 h in a shaking incubator. Next, Erlenmeyer flasks containing 50 ml of TSB were inoculated with 0·05 ml of culture. The flasks were incubated with agitation at 37°C until the stationary growth phase was reached.

HHP treatment

Bacterial cells were harvested by centrifugation at 10 000 ***g*** for 5 min at 4°C and pellets resuspended in citrate-phosphate buffer at pH 4·0. Where indicated, sodium nitrite was added to the buffer at a concentration of 1·0, 2·0 or 3·0 mmol l^{-1} with *E. coli* and 0·25, 0·50 or 0·75 mmol l^{-1} with *L. monocytogenes*. These concentrations were chosen from those previously reported (Dykhuizen *et al.* 1996). Cell suspensions were diluted in appropriate buffer to approximately 10^8 CFU ml^{-1} and 1 ml volumes placed in sterile high-density polyethylene plastic pouches (2 cm × 5 cm and 65 μm film thickness) (Seward Ltd, Worthing, West Sussex, UK) that were heat-sealed and kept on ice before pressurization. Samples were pressurized in a 300-ml pressure vessel (model S-FL-850-9-W; Stansted Fluid Power, Stansted, UK) at 18°C. The pressure-transmitting fluid was monopropylene glycol–water (30 : 70). *Escherichia coli* and *L. monocytogenes* cells were exposed to pressures of 300 and 225 MPa for 8 min, respectively. These treatment conditions were selected from preliminary work (data not shown) to identify pressure intensity and treatment time that would yield reasonably similar degrees of inactivation and injury. The maximum temperature reached during pressurization was 28°C. After decompression, the pouches were removed from the unit and placed on ice. Viable counts were determined immediately (within approximately 10 min, designated '0 h') and after 1 and 24 h of storage at 4°C.

Counts of viable cells

Samples were appropriately diluted in maximum recovery diluent (Oxoid CM 733), and 0·02 ml volumes were spread on tryptone soya agar (Oxoid CM131) supplemented with 0·3% yeast extract (TSA-YE) and 0·1% sodium pyruvate. Plates were incubated at 37°C for 24 h for *E. coli* or for 48 h for *L. monocytogenes*. At least three independent experiments were carried out.

Statistical analysis

One-way analyses of variance were carried out using SPSS WIN 12.0 software with treatment and time as main effects. The significance of differences between means was assessed by Tukey test for a confidence interval of 95%.

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4. Discusión

La presente tesis doctoral está enmarcada dentro de una línea de investigación cuyo objetivo es conseguir la máxima seguridad microbiológica de alimentos listos para el consumo (RTE) a través de la optimización de procesos de control y descontaminación, en este caso de productos cárnicos RTE, mediante tratamientos combinados de altas presiones y bioconservación. Para las investigaciones realizadas se han seleccionado dos matrices cárnicas, el jamón curado loncheado y el carpaccio de ternera. Se estima que los resultados derivados de estas investigaciones podrían ser aplicables a otros productos cárnicos afines. Se han evaluado, además, los efectos de los tratamientos y de las condiciones ensayadas sobre las características fisicoquímicas, reológicas y de color del jamón curado y del carpaccio de ternera. Se intenta no solo conseguir que los tratamientos garanticen la seguridad del producto, sino que también maximicen su calidad, puesto que un alimento seguro que no sea aceptable por el consumidor no lo será tampoco a nivel industrial y comercial.

El jamón curado loncheado y el carpaccio de ternera difieren en su actividad de agua (a_w), propiedad fisicoquímica relacionada con la estabilidad del alimento que determina la capacidad de crecimiento de los microorganismos (Rahman y Labuza, 2007). El jamón curado loncheado posee una a_w<0.90 (habitualmente oscila entre 0.80 y 0.85, en función del grado de salado y del grado de secado o tiempo de maduración) y un elevado contenido en NaCl, siendo un producto estable, con una vida útil larga a temperaturas de refrigeración (superior a tres meses). Por el contrario, el carpaccio de ternera, elaborado a partir de piezas de carne, crudas o curadas, congeladas, loncheadas, envasadas a vacío o en atmósferas modificadas y almacenadas a temperaturas de refrigeración, es un producto más perecedero con una vida útil más corta y una a_w>0.92.

Los productos crudos de carne poseen riesgos microbiológicos asociados principalmente a los patógenos *Salmonella* spp., *Escherichia coli* O157:H7 y *Listeria monocytogenes* (FDA, 2009). *S*. Enteritidis y *E. coli* verotoxigénico (VTEC) a través del consumo de carne de ternera representan un riesgo medio para la salud pública (Blagojevic y Antic, 2014). En el caso de *E. coli* verotoxigénico, este riesgo recae en la severidad de la enfermedad causada, pudiendo derivar en síndrome urémico hemolítico y colitis hemorrágica, en una mayor asociación del consumo de carne cruda con la enfermedad, considerándose una fuente importante de transmisión de VTEC (Kosmisder *et al*., 2010; Rhoades *et al*., 2009) y en la prevalencia media de VTEC en las canales de ternera (1.29%) en mataderos de vacuno, muy variable entre los Estados Miembros de la UE (EFSA/ECDC, 2012). En los años 2009 y 2010, la carne de ternera fue la causa del 3.1% y 4.7% de los brotes de salmonelosis

acontecidos en la UE, respectivamente (EFSA/ECDC, 2011, 2012), aunque recientemente esta incidencia ha disminuido (Domingues *et al*., 2012).

Por el contrario, el jamón curado loncheado, por sus características intrínsecas, no está generalmente asociado con problemas de seguridad alimentaria (Reynolds *et al*., 2001). No obstante, las exigencias sanitarias establecidas por países como EE.UU., Japón y Corea para las importaciones de productos crudos curados entre los que se encuentra el jamón curado, otros productos cárnicos RTE, o productos crudos fermentados, pueden convertirse en barreras comerciales debido a la aplicación de criterios microbiológicos estrictos como ausencia de *L. monocytogenes* en 25 g de producto (Nunes *et al*., 2011). Es importante señalar que tanto el jamón curado loncheado como el carpaccio de ternera pueden resultar contaminados con microorganismos patógenos durante las operaciones de loncheado y envasado, aumentando el riesgo de crecimiento de los patógenos durante el período de almacenamiento o distribución, si se dan condiciones de abuso o aumento incontrolado de temperatura (Cava *et al*., 2009; Garrido *et al*., 2010), constituyendo un riesgo para los consumidores y un problema para la industria alimentaria. Entre los microorganismos patógenos más peligrosos que pueden alcanzar estos productos durante su preparación, se encuentran varios serovares de *Salmonella* spp., de *E. coli* (incluido el O157:H7), *L. monocytogenes* y *Staphylococcus aureus*. Sin embargo, *L. monocytogenes* alcanza especial relevancia por su carácter ubicuo y su presencia como bacteria residente en muchas instalaciones, por ser una bacteria psicrotrofa, que puede multiplicarse si las condiciones son favorables, durante el almacenamiento del alimento RTE en refrigeración, pudiendo alcanzar niveles peligrosos. Los alimentos RTE se consumen directamente sin tratamiento higienizante o con un procesado mínimo, de ahí que adquieran especial importancia desde el punto de vista de la salud pública especialmente en grupos de población más vulnerables, como mujeres embarazadas, niños y ancianos.

Por las razones anteriormente expuestas, se han investigado tratamientos para eliminar *S.* Enteritidis, *E. coli* y *L. monocytogenes* inoculados en la superficie de los dos productos cárnicos que se almacenaron a 8 ºC, una temperatura de abuso moderado encontrada frecuentemente en los refrigeradores domésticos (Garrido *et al*., 2010), durante 60 y 30 d de refrigeración, en el caso del jamón curado y del carpaccio de ternera, respectivamente. Para conseguir la higienización de estos dos productos cárnicos se han empleado las altas presiones hidrostáticas individualmente o en combinación con bioconservantes. Esta estrategia ha sido propuesta como tecnología de barrera en la inactivación bacteriana (Leistner *et al*., 2000) para aumentar la seguridad microbiológica de alimentos. En este contexto y con el

objetivo de potenciar posibles sinergias antimicrobianas, reducir la intensidad de los tratamientos y su impacto en la calidad de los productos, se han aplicado altas presiones de forma individual o en combinación con diversos agentes biológicos con actividad antimicrobiana (bacteriocinas, sistema lactoperoxidasa (SLP) y lactoferrina (LF)).

4.1. TRATAMIENTOS EN JAMÓN CURADO LONCHEADO

4.1.1. TRATAMIENTOS COMBINADOS EN LA INACTIVACIÓN DE *L. monocytogenes*, *S.* Enteritidis y *E. coli* EN JAMÓN CURADO LONCHEADO

Con el objetivo de inactivar *S. enterica* serovar Enteritidis se aplicaron tratamientos de altas presiones a 400, 500 y 600 MPa durante 5 min a 12 ºC en jamón curado loncheado (Tabla 1).

Tabla 1. Niveles (log ufc/g) de *S.* Enteritidis en jamón curado tratado con altas presiones a 400, 500 y 600 MPa durante 5 min, mantenido durante 60 d a 8 ºC.

Tiempo	Control	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
8 h	5.48 ± 0.04^{cD}	4.42 ± 0.14^{cD}	2.94 ± 1.52^{b}	1.16 ± 1.28^{aA}
1 d	5.53 ± 0.14^{cD}	3.91 ± 0.28^{bD}	1.73 ± 1.18^{a}	1.36 ± 0.10^{aA}
3 d	5.29 ± 0.05^{dCD}	4.05 ± 0.30^{cD}	2.34 ± 0.19^{b}	1.10 ± 0.17^{aA}
7 d	4.69 ± 0.35^{dB}	3.23 ± 0.50^{cC}	1.71 ± 0.65^{b}	PRE^{aA}
15 d	4.86 ± 0.35^{dBC}	3.15 ± 0.20^{cC}	1.84 ± 0.94^{b}	PRE^{aA}
30 d	4.74 ± 0.11^{dB}	3.15 ± 0.25^{cC}	2.20 ± 0.28^{b}	PRE^{aA}
45 d	4.12 ± 0.62^{cA}	2.49 ± 0.26^{bB}	1.92 ± 0.66^{b}	PRE^{aA}
60 d	3.99 ± 0.17^{cA}	1.43 ± 0.51^{bA}	1.33 ± 0.35^{b}	PRE^{aA}

PRE, presencia en 20 g. Límite de detección 10 ufc/g. Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

De los resultados obtenidos en este estudio se comprobó la disminución gradual de los niveles de *Salmonella* en las muestras de jamón no presurizadas a lo largo del período de refrigeración investigado, siendo incapaz de crecer a 8 ºC debido a la baja a_w y alto contenido de NaCl existentes en el jamón curado. Los tratamientos de alta presión aceleraron la inactivación del patógeno y las diferencias detectadas en los recuentos aumentaron después de 7 d de almacenamiento, alcanzándose mayores reducciones logarítmicas con los tratamientos de presión más intensos. A partir de entonces y hasta el final del período investigado, *Salmonella* no se detectó por recuento directo en el jamón tratado a 600 MPa, aunque sí se determinó su presencia en 20 g del producto tras realizar el enriquecimiento de las muestras. Las células

dañadas por la presurización no fueron capaces de recuperarse durante el período de almacenamiento a 8 ºC. Teniendo en cuenta que los niveles esperados de contaminación por *Salmonella* en jamón curado son bajos, y que inmediatamente después de la aplicación de 600 MPa durante 5 min se consiguieron reducciones superiores a 4 unidades logarítmicas, este tratamiento podría considerarse efectivo para conseguir un producto seguro, incluso después del almacenamiento bajo condiciones de abuso de temperatura.

Con el objetivo de aplicar tratamientos de presurización menos intensos se investigaron presiones de 400 y 500 MPa durante 10 min en combinación con dos bacteriocinas comerciales, pediocina y nisina sobre *E. coli* O157:H7. Al ser *E. coli* O157:H7 considerado un serotipo más resistente a la presión que otros (Benito *et al*., 1999), se ensayaron tratamientos más prolongados aunque sin llegar a 600 MPa. Como se muestra en la Tabla 2 y en consonancia con lo expuesto anteriormente, la pérdida de viabilidad de *E. coli* O157:H7 detectada por el efecto inmediato de la presurización fue baja, alcanzándose reducciones de 0.25 y 1.28 unidades logarítmicas con los tratamientos aplicados.

Por otro lado, la aplicación individual de pediocina o nisina no afectó a la supervivencia del patógeno. Las bacterias Gram-negativas no son sensibles a algunos agentes antimicrobianos como la lisozima, nisina u otras bacteriocinas de bacterias lácticas (Hauben *et al*., 1996), pero si estos compuestos se añaden antes de la aplicación de los tratamientos de presurización, la sensibilidad de las células microbianas a estos agentes aumenta (Hauben *et al*., 1996; Kalchayanand *et al*., 1998), como consecuencia del daño originado en la membrana externa por las altas presiones.

En el presente trabajo no se detectó un aumento de la actividad antimicrobiana con la combinación de la pediocina con los tratamientos de alta presión, a diferencia de lo que ocurrió con la combinación con nisina. La inactivación alcanzada por la combinación de nisina con la presurización fue superior a la inactivación obtenida individualmente, detectándose un efecto antimicrobiano sinérgico que se mantuvo con el tratamiento de 500 MPa combinado con nisina durante los 60 d de almacenamiento a 8 ºC.

Tabla 2. Niveles (log ufc/g) de *E. coli* O157:H7 en jamón curado presurizado a 400 y 500 MPa durante 10 min, nisina, pediocina y sus combinaciones, mantenido durante 60 d a 8 ºC.

	0 h	8 h	1 d	7 d	15 d	30 d	45 d	60 d
Control	6.13 ± 0.28^{eBC}	6.25 ± 0.05^{dC}	6.16 ± 0.30^{fC}	6.27 ± 0.17^{fC}	5.93 ± 0.06^{dB}	5.93 ± 0.13^{efB}	5.66 ± 0.51^{eA}	5.70 ± 0.38^{dA}
400 MPa	5.88 ± 0.10^{dF}	5.39 ± 0.68^{cE}	4.44 ± 0.71^{dD}	3.47 ± 0.65^{bcC}	3.16 ± 0.59^{bBC}	2.94 ± 0.73^{bcB}	2.95 ± 1.05^{cB}	2.42 ± 0.68^{bA}
500 MPa	4.85 ± 0.05^{bF}	4.90 ± 0.51^{bF}	4.07 ± 0.30^{abE}	3.54 ± 0.76^{bcD}	2.99 ± 0.13^{bC}	2.71 ± 0.37^{bBC}	2.30 ± 0.49^{abA}	2.52 ± 0.71^{bAB}
Nisina	6.42 ± 0.15^{fE}	6.22 ± 0.20^{dD}	6.19 ± 0.06^{fD}	5.86 ± 0.11^{eBC}	6.00 ± 0.12^{dC}	6.00 ± 0.03^{fC}	5.70 ± 0.22^{eB}	5.39 ± 0.15^{dA}
Pediocina	6.10 ± 0.31^{eCD}	6.08 ± 0.33^{dCD}	6.13 ± 0.20^{fD}	5.97 ± 0.07^{efC}	5.75 ± 0.43^{dB}	5.67 ± 0.26^{eAB}	5.97 ± 0.11^{eC}	5.57 ± 0.10^{dA}
400 MPa+Nis	4.81 ± 0.06^{bE}	5.21 ± 0.25^{cF}	4.31 ± 0.26^{cdD}	3.73 ± 0.31^{cdC}	3.18 ± 0.61^{bB}	3.37 ± 0.43^{dB}	2.45 ± 0.19^{bA}	2.63 ± 0.69^{bA}
400 MPa+Ped	5.96 ± 0.17^{dE}	5.36 ± 0.24^{cD}	5.03 ± 0.41^{eC}	3.40 ± 0.14^{bB}	3.23 ± 0.73^{bcAB}	3.24 ± 1.29^{cdAB}	3.39 ± 0.61^{dAB}	3.09 ± 0.83^{cA}
500 MPa+Nis	4.32 ± 0.56^{aD}	4.28 ± 0.20^{aD}	4.00 ± 0.11^{aD}	3.01 ± 0.06^{aC}	2.55 ± 0.64^{aB}	2.06 ± 0.29^{aA}	2.01 ± 0.28^{aA}	1.70 ± 0.83^{aA}
500 MPa+Ped	5.03 ± 0.05^{cG}	4.79 ± 0.50^{bF}	4.22 ± 0.11^{bcE}	3.98 ± 0.51^{dD}	3.47 ± 0.24^{cC}	2.94 ± 0.13^{bcB}	2.59 ± 0.29^{bA}	2.64 ± 0.32^{bcA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Las células de *E. coli* dañadas por las altas presiones, no solamente no fueron capaces de recuperarse durante el período de refrigeración investigado sino que disminuyeron 3.46 logs en las tratadas a 400 MPa y 2.33 logs en las tratadas a 500 MPa. En las muestras no presurizadas, los recuentos de *E. coli* disminuyeron 0.43 unidades logarítmicas durante 60 d a 8 ºC, indicando la capacidad de supervivencia del patógeno en jamón curado durante un período de almacenamiento prolongado.

Las condiciones de baja a_w y alto contenido en NaCl presentes en el jamón curado constituyen un ambiente adverso para la recuperación de las células estresadas de distintos patógenos (De Alba *et al*., 2012a, 2013a; Bover-Cid *et al*., 2011). Se han comprobado en distintos trabajos diferencias en la inactivación de *E. coli* en diversos productos cárnicos, atribuibles a las distintas características fisicoquímicas de estos alimentos (Palou *et al*., 1997). Es interesante señalar que valores bajos de a_w ejercen un efecto baroprotector frente a los microorganismos (Oxen y Knorr, 1993; Patterson, 2005; Hereu *et al*., 2012).

Otra combinación investigada en la presente tesis doctoral fue la de alta presión a 450 MPa durante 10 min, el sistema lactoperoxidasa (SLP) o lactoferrina (LF) frente a *L. monocytogenes* y *S*. Enteritidis inoculados en jamón curado loncheado, cuyos resultados se muestran en las Tablas 3 y 4.

Las condiciones de presurización fueron elegidas en función de los resultados obtenidos por Morales *et al*. (2006), quienes observaron reducciones de 1.16 unidades logarítmicas en *L. monocytogenes* Scott A inoculada en jamón Serrano loncheado, tras la aplicación de 450 MPa durante 10 min. En el presente trabajo, las reducciones de *L. monocytogenes* INIA H66a alcanzadas después de la presurización fueron de 0.58 unidades logarítmicas, inferiores a las detectadas por Morales *et al*. (2006). Estos resultados ponen de manifiesto la variabilidad existente entre distintas cepas de *L. monocytogenes* en respuesta a las altas presiones (Murano *et al*., 1999; Tay *et al*., 2003) y la mayor sensibilidad de una cepa de colección, la Scott A, frente a una cepa residente procedente de la industria cárnica. Sin embargo, las reducciones de *S*. Enteritidis fueron superiores, mostrando la menor barotolerancia experimentada en general por los Gram-negativos. La aplicación individual de los dos bioconservantes ensayados, LF y el SLP, no afectó a los recuentos de los dos patógenos. Esta ausencia de actividad podría estar relacionada con la interferencia de ambos bioconservantes con los componentes o con los agentes del curado del jamón, o con el tamaño del inóculo, como habían observado previamente Kennedy *et al*. (2000) en carne picada tratada con el SLP o Al-Nabulsi y Holley (2007) en embutidos tratados con LF. Sin embargo, Colak *et al*. (2008) detectaron reducciones

significativas de *E. coli* y *Pseudomonas* spp. inoculados en albóndigas de carne mediante la aplicación individual de LF. Por otro lado, Montiel *et al*. (2012) observaron que la adición del SLP a salmón ahumado reducía los recuentos de *L. monocytogenes* INIA H66a en 1 unidad logarítmica, aunque el patógeno reiniciaba su crecimiento en muestras refrigeradas a 5 ºC a partir de 7 d de almacenamiento. La actividad antimicrobiana del SLP, ejercida a través de la oxidación de grupos sulfhidrilo de enzimas y otras proteínas presentes en las membranas bacterianas, fue muy reducida en el jamón curado con descensos de 0.2 y 0.01 unidades logarítmicas en los recuentos de *L. monocytogenes* y de *S*. Enteritidis, respectivamente, tras la aplicación del tratamiento. Respecto a la actividad de la LF, la presencia de cationes en la matriz alimentaria, podría interferir impidiendo la acción antimicrobiana en jamón curado como indicaron Del Olmo *et al*. (2009) en carne picada. La actividad bactericida de la LF parece estar basada en interacciones electrostáticas con las moléculas de lipopolisacárido de la membrana externa de los Gram-negativos (Ellison, 1994) o con los ácidos teicoicos o lipoteicoicos de los Gram-positivos (Vorland *et al*., 1999), causando despolarización, pérdida de la integridad de membrana y del gradiente de protones.

Cuando se aplicaron tratamientos combinados de alta presión y LF o el SLP en jamón curado loncheado, se observó un efecto antimicrobiano sinérgico sobre *S*. Enteritidis, alcanzándose reducciones logarítmicas 1.45 y 2.09 mayores que la suma de la inactivación obtenida con cada tratamiento individual. Este efecto bactericida aumentó a partir de los días 1 y 15 de almacenamiento a 8 ºC, de forma que el patógeno solo fue detectado mediante el enriquecimiento de las muestras.

Como se ha indicado anteriormente, el efecto sinérgico entre altas presiones y algunos antimicrobianos ha sido atribuido a la permeabilización por la presurización de la membrana externa de las bacterias, permitiendo que estos compuestos alcancen la pared celular o la membrana plasmática y puedan ejercer su actividad bactericida. Frente a *L. monocytogenes* se observó un ligero efecto antimicrobiano sinérgico con la combinación de la alta presión y el SLP al final del período de almacenamiento. Por el contrario, la presurización combinada con LF no aumentó la inactivación de este patógeno. Montiel *et al*. (2012) también observaron que el tratamiento de 450 MPa durante 10 min en combinación con el SLP causaba mayor letalidad frente a *L. monocytogenes* en salmón ahumado que la aplicación individual de los tratamientos, indicando un efecto antimicrobiano sinérgico principalmente al final del período de refrigeración. Por otra parte, el efecto antimicrobiano de la LF y sus derivados en combinación con el tratamiento de 400 MPa durante 10 min sobre

L. monocytogenes inoculada en carne de pollo resultó muy leve (Del Olmo *et al*., 2012a).

Los recuentos de ambos patógenos (Tablas 3 y 4) también disminuyeron en las muestras no tratadas durante el almacenamiento a 8 ºC, aunque *S*. Enteritidis resultó más afectado por las condiciones del jamón curado que *L. monocytogenes*, mostrando este último mayor capacidad para sobrevivir en este producto durante un almacenamiento prolongado. Según la ICMSF (1996), las características intrínsecas del jamón curado no favorecen el crecimiento de *L. monocytogenes* en el caso de una contaminación accidental pero permiten su supervivencia. El Reglamento CE (2007) define las condiciones en que *L. monocytogenes* no se multiplica en refrigeración, pH≤4.4 o a_w≤0.92, o pH≤5.0 y a_w≤0.94. El pH influye poco en el crecimiento de esta bacteria pero la a_w es crítica.

Tabla 3. Niveles (log ufc/g) de *L. monocytogenes* en jamón curado tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF y sus combinaciones, mantenido durante 60 d a 8 ºC.

	0 h	1 d	15 d	30 d	60 d
Control	5.27 ± 0.12^{cC}	5.34 ± 0.09^{cC}	5.25 ± 0.14^{cBC}	5.14 ± 0.14^{dB}	4.87 ± 0.31^{cA}
SLP	5.07 ± 0.35^{bB}	5.20 ± 0.10^{bcC}	5.11 ± 0.17^{bBC}	5.10 ± 0.12^{dBC}	4.91 ± 0.13^{cA}
LF	5.39 ± 0.11^{dBC}	5.47 ± 0.43^{cC}	5.31 ± 0.23^{cABC}	5.13 ± 0.19^{dAB}	5.02 ± 0.09^{dA}
450 MPa	4.69 ± 0.12^{aC}	4.90 ± 0.18^{abD}	4.41 ± 0.09^{aB}	4.34 ± 0.33^{cB}	4.23 ± 0.18^{bA}
450 MPa + SLP	4.72 ± 0.12^{aD}	4.91 ± 0.10^{abE}	4.51 ± 0.12^{aC}	4.15 ± 0.36^{aB}	4.01 ± 0.28^{aA}
450 MPa + LF	4.69 ± 0.15^{aD}	4.70 ± 0.22^{aD}	4.49 ± 0.09^{aC}	4.26 ± 0.42^{bA}	4.31 ± 0.12^{bB}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$.

Tabla 4. Niveles (log ufc/g) de *S.* Enteritidis en jamón curado tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF y sus combinaciones, mantenido durante 60 d a 8 ºC.

	0 h	1 d	15 d	30 d	60 d
Control	4.67 ± 0.69^{dA}	4.71 ± 0.58^{dA}	3.87 ± 1.83^{bA}	3.12 ± 2.05^{bA}	2.73 ± 1.87^{bA}
SLP	4.66 ± 0.31^{dB}	4.79 ± 0.31^{dB}	4.41 ± 0.53^{bB}	3.47 ± 1.59^{bAB}	2.64 ± 1.86^{bA}
LF	4.59 ± 0.64^{dBC}	4.68 ± 0.36^{dC}	4.53 ± 0.50^{bBC}	2.89 ± 1.88^{bAB}	2.61 ± 1.41^{bA}
450 MPa	2.45 ± 0.60^{cD}	1.35 ± 0.44^{cC}	0.60 ± 0.24^{aB}	0.08 ± 0.14^{aA}	PRE^{aA}
450 MPa + SLP	0.35 ± 0.37^{aB}	PRE^{aA}	PRE^{aA}	PRE^{aA}	PRE^{aA}
450 MPa + LF	0.92 ± 0.08^{bC}	0.25 ± 0.31^{bB}	PRE^{aA}	PRE^{aA}	PRE^{aA}

PRE, presencia en 20 g. Límite de detección 10 ufc/g. Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$.

En cuanto a los niveles de viables totales, como se muestra en la tabla 5, no resultaron afectados por la aplicación individual de la LF o del SLP en el jamón curado. Sin embargo, Colak *et al*. (2008), detectaron que la aplicación individual de LF en albóndigas producía una reducción significativa de aerobios totales. También Kennedy *et al*. (2000) observaron un efecto bacteriostático del SLP sobre la mayoría de las poblaciones microbianas presentes en carne picada de ternera, mientras que Elliot *et al*. (2004) detectaron un efecto bacteriostático sobre las pseudomonas pero no sobre la microbiota láctica endógena de la carne de ternera. Según Seifu *et al*. (2005), el efecto antimicrobiano del SLP depende de las especies bacterianas que están presentes y de las condiciones del medio, pH y temperatura. En el presente trabajo, los niveles de viables totales en el jamón curado fueron menores en las muestras presurizadas y en las que se aplicaron tratamientos combinados que en las muestras control y en las que contenían LF y el SLP individualmente. Al final del período de almacenamiento se observó un aumento de viables totales en todas las muestras, aunque en las tratadas con la combinación de altas presiones y el SLP se registró un efecto antimicrobiano sinérgico de 2.19 unidades logarítmicas. También Montiel *et al*. (2012) obtuvieron resultados similares en salmón ahumado presurizado a 250 MPa y 450 MPa durante 10 min, el SLP y sus combinaciones, con un aumento de viables totales en salmón ahumado durante 35 d de refrigeración a 5 ºC. Estos autores observaron que el tratamiento de 450 MPa y el SLP mantenía los niveles de viables totales por debajo del límite de detección durante el período investigado.

Tabla 5. Niveles (log ufc/g) de viables totales en jamón curado tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF, y sus combinaciones, mantenido durante 60 d a 8 ºC.

	1 d	30 d	60 d
Control	3.59 ± 0.19^{bA}	4.22 ± 0.26^{bB}	5.07 ± 0.24^{bcC}
SLP	3.40 ± 0.46^{bA}	4.21 ± 0.14^{bB}	5.21 ± 1.05^{dC}
LF	3.16 ± 1.28^{bA}	4.48 ± 0.22^{bB}	5.17 ± 0.28^{cdB}
450 MPa	2.07 ± 0.34^{aA}	3.00 ± 0.65^{aB}	5.03 ± 0.24^{bC}
450 MPa + SLP	2.18 ± 0.33^{aA}	3.18 ± 0.61^{aB}	2.88 ± 0.56^{aB}
450 MPa + LF	2.11 ± 0.35^{aA}	2.83 ± 1.21^{aB}	4.95 ± 0.39^{bC}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$.

4.1.2. EFECTO DE LOS TRATAMIENTOS SOBRE LAS CARACTERÍSTICAS DEL JAMÓN CURADO LONCHEADO

De los estudios realizados en jamón curado loncheado se desprende que los tratamientos de presurización aumentaron ligeramente el pH de las muestras, aunque las diferencias con el jamón control tendieron a atenuarse al final del período de refrigeración como se muestra en las Tablas 6, 7 y 8. Este aumento ha sido atribuido a la desnaturalización proteica originada por las altas presiones que como resultado, y debido al desplegamiento proteico, deja a los aminoácidos básicos expuestos al medio, o a la pérdida de protones causada por la presurización (Fulladosa *et al*., 2009; Marcos *et al*., 2005). También se observó un ligero aumento de pH en jamón tratado con LF, efecto ya registrado en otros productos cárnicos tratados con LF, nisina y sus combinaciones (Colak *et al*., 2008). Los valores de a_w también aumentaron en el jamón presurizado (Tablas 6, 7 y 8), aunque las diferencias no fueron suficientes para comprometer la seguridad microbiológica del producto. Serra *et al*. (2007) observaron un aumento de los valores de a_w en jamones presurizados, con tiempos de maduración más cortos, aunque las diferencias también fueron pequeñas.

Tabla 6. Valores de pH y a_w del jamón curado tratado con altas presiones a 400, 500 y 600 MPa durante 5 min, mantenido durante 60 d a 8 ºC.

		Control	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
pH	1 d	5.85 ± 0.00^{aB}	5.89 ± 0.01^{bC}	5.91 ± 0.00^{bC}	5.91 ± 0.01^{bC}
	15 d	5.74 ± 0.01^{aA}	5.69 ± 0.04^{aA}	5.71 ± 0.01^{aA}	5.79 ± 0.08^{aAB}
	30 d	5.75 ± 0.01^{bA}	5.70 ± 0.00^{aA}	5.75 ± 0.02^{bA}	5.78 ± 0.01^{bA}
	45 d	5.92 ± 0.04^{aC}	5.93 ± 0.03^{aC}	5.95 ± 0.02^{aC}	5.92 ± 0.02^{aC}
	60 d	5.85 ± 0.01^{aB}	5.84 ± 0.02^{aB}	5.83 ± 0.01^{aB}	5.86 ± 0.02^{aBC}
a_w	1 d	0.891 ± 0.002^{aA}	0.896 ± 0.002^{abB}	0.899 ± 0.002^{bAB}	0.887 ± 0.004^{abA}
	15 d	0.886 ± 0.004^{aA}	0.892 ± 0.002^{bA}	0.897 ± 0.002^{cA}	0.901 ± 0.002^{dB}
	30 d	0.886 ± 0.007^{aA}	0.897 ± 0.003^{bBC}	0.901 ± 0.003^{bBC}	0.901 ± 0.002^{bB}
	45 d	0.887 ± 0.005^{aA}	0.899 ± 0.003^{bBC}	0.901 ± 0.003^{bcBC}	0.903 ± 0.002^{cB}
	60 d	0.891 ± 0.005^{aA}	0.900 ± 0.002^{bC}	0.903 ± 0.001^{bC}	0.901 ± 0.003^{bB}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 7. Valores de pH y a_w del jamón curado tratado con altas presiones a 400 y 500 MPa durante 10 min, nisina, pediocina y sus combinaciones, mantenido durante 60 d a 8 ºC.

		1 d	30 d	60 d
pH	Control	5.87 ± 0.05^{bcC}	5.68 ± 0.04^{abA}	5.82 ± 0.06^{bcdeB}
	Nisina	5.84 ± 0.02^{abB}	5.76 ± 0.04^{cA}	5.78 ± 0.09^{abA}
	Pediocina	5.94 ± 0.16^{eB}	5.83 ± 0.14^{dA}	5.85 ± 0.04^{efA}
	400 MPa	5.93 ± 0.06^{deC}	5.76 ± 0.03^{cA}	5.83 ± 0.11^{cdeB}
	500 MPa	5.90 ± 0.02^{cdC}	5.78 ± 0.13^{cdA}	5.84 ± 0.02^{deB}
	400 MPa + Nis	5.84 ± 0.02^{abB}	5.75 ± 0.07^{cA}	5.78 ± 0.05^{abcA}
	400 MPa + Ped	5.81 ± 0.06^{aA}	5.76 ± 0.11^{cA}	5.79 ± 0.05^{bcdA}
	500 MPa + Nis	5.87 ± 0.05^{bcC}	5.64 ± 0.02^{aA}	5.73 ± 0.10^{aB}
	500 MPa + Ped	5.86 ± 0.06^{bcB}	5.72 ± 0.11^{bcA}	5.90 ± 0.15^{fB}
a_w	Control	0.884 ± 0.009^{aAB}	0.887 ± 0.004^{aB}	0.882 ± 0.011^{aA}
	Nisina	0.899 ± 0.006^{cA}	0.897 ± 0.005^{bcA}	0.906 ± 0.008^{efB}
	Pediocina	0.893 ± 0.005^{bA}	0.897 ± 0.006^{bcB}	0.904 ± 0.007^{deC}
	400 MPa	0.903 ± 0.004^{deC}	0.895 ± 0.005^{bA}	0.897 ± 0.006^{bB}
	500 MPa	0.904 ± 0.007^{eC}	0.897 ± 0.004^{cA}	0.901 ± 0.005^{cB}
	400 MPa + Nis	0.904 ± 0.007^{eB}	0.901 ± 0.012^{deA}	0.907 ± 0.006^{fC}
	400 MPa + Ped	0.901 ± 0.011^{cdAB}	0.900 ± 0.006^{dA}	0.902 ± 0.009^{cdB}
	500 MPa + Nis	0.900 ± 0.012^{cdA}	0.902 ± 0.008^{eB}	0.907 ± 0.008^{fC}
	500 MPa + Ped	0.901 ± 0.013^{cdA}	0.902 ± 0.009^{eA}	0.901 ± 0.002^{cA}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$.

Tabla 8. Valores de pH y a_w del jamón curado tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF y sus combinaciones, mantenido durante 60 d a 8 ºC.

		1 d	30 d	60 d
pH	Control	5.84 ± 0.05^{bA}	6.08 ± 0.10^{aB}	6.05 ± 0.12^{bB}
	SLP	5.84 ± 0.04^{bA}	6.33 ± 0.34^{aB}	5.96 ± 0.07^{aAB}
	LF	5.93 ± 0.08^{cA}	6.07 ± 0.09^{aB}	5.96 ± 0.05^{aA}
	450 MPa	5.88 ± 0.08^{bA}	6.24 ± 0.07^{aC}	6.12 ± 0.06^{cB}
	450 MPa + SLP	5.69 ± 0.08^{aA}	6.25 ± 0.12^{aC}	6.00 ± 0.12^{abB}
	450 MPa + LF	5.68 ± 0.03^{aA}	6.14 ± 0.06^{aC}	6.00 ± 0.04^{abB}
a_w	Control	0.865 ± 0.006^{aA}	0.866 ± 0.004^{aA}	0.870 ± 0.005^{abA}
	SLP	0.873 ± 0.004^{bA}	0.874 ± 0.002^{cA}	0.872 ± 0.005^{bA}
	LF	0.871 ± 0.003^{bB}	0.870 ± 0.002^{bB}	0.866 ± 0.008^{aA}
	450 MPa	0.876 ± 0.003^{cB}	0.874 ± 0.003^{cB}	0.869 ± 0.005^{abA}
	450 MPa + SLP	0.878 ± 0.001^{cB}	0.879 ± 0.004^{dB}	0.871 ± 0.003^{bA}
	450 MPa + LF	0.877 ± 0.002^{cC}	0.875 ± 0.002^{cB}	0.871 ± 0.005^{bA}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$.

En el jamón presurizado a 400, 500 y 600 MPa durante 5 min, se detectó un aumento de los valores de TBA-RS (sustancias reactivas con el ácido tiobarbitúrico) después de 30 y 60 d de refrigeración a 8 ºC (Tabla 9).

Tabla 9. Valores de TBA-RS (mg MDA/kg) en jamón tratado con altas presiones a 400, 500 y 600 MPa durante 5 min, mantenido durante 60 d a 8 ºC.

	Control	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
1 d	0.38 ± 0.01^{aA}	0.36 ± 0.01^{aA}	0.40 ± 0.00^{aA}	0.35 ± 0.07^{aA}
30 d	0.46 ± 0.02^{aB}	0.51 ± 0.01^{dC}	0.47 ± 0.01^{bB}	0.49 ± 0.01^{cB}
60 d	0.49 ± 0.01^{bC}	0.45 ± 0.10^{aB}	0.53 ± 0.00^{dC}	0.52 ± 0.01^{cB}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Los valores de TBA-RS tendían a aumentar durante la refrigeración, en consonancia con los resultados obtenidos por Cava *et al*. (2009) en jamón curado loncheado envasado a vacío, presurizado a 200-300 MPa durante 15-30 min y almacenado en refrigeración a 4 ºC durante 90 d. También Andrés *et al*. (2006) detectaron valores mayores de TBA-RS en jamón curado presurizado a 400 MPa y almacenado durante

39 d, mostrando una menor estabilidad oxidativa durante la refrigeración. Por el contrario, en lomo curado se observaron resultados opuestos, con valores mayores de TBA-RS en las muestras no presurizadas después de 1 día de almacenamiento (Campus *et al*., 2008), aunque estas diferencias se atenuaron durante el período de refrigeración. Según Andrés *et al.* (2004), las altas presiones podrían promover la oxidación lipídica por rotura de los pigmentos hemo, liberando hierro ferroso con efecto catalítico en la oxidación lipídica, o por la desintegración de las membranas, haciendo a los ácidos grasos poliinsaturados susceptibles a la oxidación. Este efecto prooxidante ha sido también observado por Cava *et al*. (2009) en jamón Ibérico loncheado. En general, los cambios en la oxidación lipídica de los productos cárnicos curados loncheados debidos a la presurización fueron menores que los cambios producidos por el almacenamiento en refrigeración.

En cuanto al contenido en aminoácidos libres, se observaron cambios ligeros en jamón presurizado a 400, 500 y 600 MPa y en el jamón control a los 60 d de almacenamiento a 8 ºC, probablemente debido a que la mayor parte de la proteólisis había tenido lugar durante el curado (Tabla 10).

Tabla 10. Contenido de aminoácidos libres (mg/100 g) en jamón tratado con altas presiones a 400, 500 y 600 MPa durante 5 min, mantenido durante 60 d a 8 ºC.

	Control	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
Asp	282.0±27.8^{a}	256.1±2.5^{a}	248.7±12.0^{a}	263.3±2.7^{a}
Ser	225.9±0.8^{a}	237.7±2.0^{b}	228.9±0.5^{a}	235.4±2.2^{b}
Glu	284.9±0.8^{b}	280.3±6.2^{b}	265.2±1.7^{a}	279.6±1.6^{b}
Gly	173.7±0.8^{b}	175.4±0.2^{ab}	170.2±0.4^{a}	177.8±0.8^{c}
His	197.9±4.3^{a}	198.9±0.3^{a}	198.1±1.3^{a}	204.4±0.5^{a}
Arg	928.1±1.9^{bc}	939.6±8.0^{c}	901.7±1.8^{a}	915.2±2.3^{ab}
Thr	257.5±1.0^{ab}	259.5±0.7^{b}	253.7±1.4^{a}	260.2±0.7^{b}
Ala	352.4±0.9^{b}	356.3±1.5^{c}	348.5±0.3^{a}	357.5±0.1^{c}
Pro	249.8±1.1^{a}	236.6±2.0^{a}	237.1±8.6^{a}	241.0±10.8^{a}
Tyr	134.4±0.5^{d}	131.5±0.6^{c}	124.8±0.1^{b}	122.9±0.1^{a}
Val	306.9±0.6^{b}	308.7±0.1^{bc}	301.9±0.4^{a}	310.1±0.5^{c}
Met	114.4±1.8^{a}	118.4±0.8^{a}	115.3±0.3^{a}	118.2±0.1^{a}
Lys	557.2±5.2^{ab}	571.8±1.2^{c}	550.9±2.2^{a}	566.7±0.9^{bc}
Ile	242.1±1.1^{b}	244.1±0.1^{b}	238.9±0.3^{a}	244.5±0.5^{b}
Leu	401.8±0.7^{b}	405.8±1.2^{b}	396.2±1.1^{a}	404.4±1.1^{b}
Phe	221.7±0.1^{b}	224.3±0.1^{c}	219.5±0.5^{a}	224.5±0.8^{c}
Total	4930.5±17.3^{b}	4944.9±17.1^{b}	4799.4±16.9^{a}	4925.6±2.2^{b}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$.

Respecto al color del jamón curado presurizado y como se muestra a continuación en la Tabla 11, la luminosidad (L^*) aumentó en el jamón tratado con 500 y 600 MPa

durante 5 min en comparación con las muestras no tratadas y las presurizadas a 400 MPa, excepto 1 día después de los tratamientos, en el que se observaron valores mayores de *L** a 400 MPa. La *L** disminuyó ($P<0.001$) durante el almacenamiento en el jamón control y en el tratado, y los cambios inducidos durante el período de refrigeración fueron mayores que los inducidos por la presurización. Los valores de la tendencia al rojo (*a**) fueron menores en el jamón tratado a 500 y 600 MPa. Esta tendencia se mantuvo durante 60 d de almacenamiento a 8 ºC y los valores más bajos se obtuvieron al final del período en refrigeración. Los valores de la tendencia al amarillo (*b**) resultaron también significativamente afectados ($P<0.001$) por los tratamientos de alta presión. Después de 24 h, las muestras tratadas a 600 MPa mostraron valores significativamente menores que el resto, presurizadas y no presurizadas. La tendencia al amarillo disminuyó durante el almacenamiento ($P<0.05$), registrándose los valores más bajos en el jamón presurizado a 400 MPa y 600 MPa después de 60 d a 8 ºC.

Los tratamientos de alta presión modifican en general las propiedades del color de la carne y de los productos cárnicos. Con tratamientos entre 200 MPa y 350 MPa se produce en la carne fresca un empalidecimiento, atribuido por Carlez *et al*. (1995) a la desnaturalización de la globina y/o al desplazamiento o liberación del grupo hemo, o a la oxidación de la mioglobina ferrosa a mioglobina férrica y por consiguiente, a un aumento en la proporción de metamioglobina a expensas de la oximioglobina, a presiones superiores de 400 MPa. Según Andrés *et al*. (2006), los pigmentos rojos de los productos curados son sensibles a las altas presiones. La pérdida de la tendencia al rojo del jamón curado por tratamientos de presurización ha sido indicada previamente, detectándose los valores más bajos en los productos expuestos a las condiciones de presión más altas (Andrés *et al*., 2006; Cava *et al*., 2009; Morales *et al*., 2006; De Alba *et al*., 2012a). Sin embargo, el color de las carnes crudas curadas se ve menos afectado por esta tecnología que la carne fresca, y parece que el curado podría conferir mayor estabilidad al color del jamón (Serra *et al*., 2007) al proteger el óxido nítrico a la mioglobina de la oxidación producida por los tratamientos de alta presión, debido a la formación de nitrosilmioglobina, pigmento responsable del color de la carne curada (Moller y Skibsted, 2002). Andrés *et al*. (2004) observaron valores menores de *L** en el jamón Ibérico presurizado a 200-400 MPa, mientras que con tratamientos de 600-800 MPa, los valores de *L** fueron similares a los controles. Sin embargo, Morales *et al*. (2006) no detectaron cambios en los valores de *L**, *a** o *b** por la aplicación de tratamientos de 450 MPa durante 10 min en jamón Serrano almacenado durante 7 d a 4 ºC, aunque observaron diferencias en los valores de *L** y *a** entre diferentes puntos de la misma loncha. En lomo curado presurizado, Campus *et al*. (2008) describieron la reducción de *L** durante el almacenamiento y el

mantenimiento de las diferencias entre las muestras control y las tratadas. Sin embargo, estas diferencias se atenuaron durante el almacenamiento de jamones presurizados envasados en atmósfera modificada (Andrés *et al*., 2004). Las variaciones de la tendencia al amarillo (*b**) en los productos cárnicos ha sido relacionada con cambios en el estado químico de la mioglobina (Goutefongea *et al*., 1995). Estos autores observaron un aumento de *b** en carne fresca picada de ternera y cerdo, inducido por el tratamiento de alta presión a 600 MPa durante 30 min.

Tabla 11. Parámetros de color del jamón curado tratado con altas presiones a 400, 500 y 600 MPa durante 5 min, mantenido durante 60 d a 8 ºC.

		Control	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
*L**	1 d	47.02 ± 3.86^{aB}	49.34 ± 3.28^{bC}	47.11 ± 2.76^{aA}	46.79 ± 3.24^{aC}
	15 d	44.70 ± 2.63^{aAB}	45.67 ± 3.45^{abB}	47.24 ± 2.96^{bA}	46.54 ± 2.29^{bBC}
	30 d	43.56 ± 3.17^{aA}	43.15 ± 4.16^{aA}	46.94 ± 3.53^{bA}	44.64 ± 2.90^{abABC}
	45 d	44.97 ± 4.02^{abAB}	44.47 ± 2.72^{abAB}	45.78 ± 3.79^{bA}	43.60 ± 3.10^{aA}
	60 d	43.29 ± 3.91^{aA}	43.67 ± 2.73^{aAB}	45.77 ± 2.87^{bA}	44.42 ± 2.95^{abAB}
*a**	1 d	22.28 ± 2.02^{cC}	20.61 ± 2.96^{bB}	19.42 ± 1.64^{abA}	18.50 ± 1.47^{aAB}
	15 d	21.04 ± 1.66^{bABC}	18.95 ± 1.81^{aA}	18.46 ± 1.41^{aA}	18.81 ± 1.25^{aB}
	30 d	20.68 ± 1.92^{bAB}	20.28 ± 2.23^{bAB}	18.33 ± 1.78^{aA}	18.34 ± 1.34^{aAB}
	45 d	19.80 ± 2.29^{aA}	19.77 ± 1.34^{aAB}	18.94 ± 1.75^{aA}	19.35 ± 1.69^{aB}
	60 d	21.36 ± 2.14^{cBC}	20.22 ± 1.84^{bcAB}	19.32 ± 1.39^{bA}	17.50 ± 1.39^{aA}
*b**	1 d	19.23 ± 2.25^{bA}	19.77 ± 2.35^{bB}	18.93 ± 2.50^{bA}	16.93 ± 2.77^{aA}
	15 d	18.92 ± 3.13^{aA}	16.88 ± 3.02^{aA}	17.70 ± 2.56^{aA}	17.23 ± 3.20^{aA}
	30 d	18.37 ± 3.17^{bA}	18.48 ± 2.84^{bAB}	17.81 ± 2.39^{abA}	15.97 ± 3.24^{aA}
	45 d	17.91 ± 3.16^{aA}	18.35 ± 2.45^{aAB}	18.11 ± 2.83^{aA}	17.46 ± 3.96^{aA}
	60 d	19.93 ± 3.85^{bA}	17.42 ± 3.51^{aA}	17.73 ± 3.21^{abA}	15.64 ± 2.65^{aA}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Respecto a los cambios en el color del jamón sometido a los distintos tratamientos combinados, cuando se combinó nisina o pediocina con presurizaciones de 500 MPa durante 10 min se observó una disminución de *L** (Tabla 12). La luminosidad tendía a disminuir también en el jamón control y en el tratado durante el período en refrigeración, siendo los cambios inducidos por el almacenamiento a 8 ºC, como en el caso de los tratamientos individuales de alta presión, mayores que los originados por la presurización. Los valores de *a** resultaron ligeramente afectados por los tratamientos, tendiendo a disminuir con la refrigeración. Los valores de *b** no cambiaron significativamente en el jamón presurizado o tratado con las

bacteriocinas, con la excepción del tratamiento de 400 MPa combinado con pediocina que produjo un aumento (P<0.05) de b^*. En la mayoría de los casos, los valores más bajos de b^* se obtuvieron después de 60 d de almacenamiento en refrigeración. En general, los cambios originados en las propiedades del color del jamón curado sometido a tratamientos de 400 o 500 MPa durante 10 min, individualmente o en combinación con los bioconservantes fueron bajos, y los valores de los tres parámetros L^*, a^* y b^* tendían a disminuir durante el almacenamiento en refrigeración (De Alba *et al*., 2013a).

En el jamón curado tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF o sus combinaciones no se observaron diferencias significativas en el valor de L^*, excepto en las muestras presurizadas y combinadas con el SLP, en las que se observó un aumento (P<0.05) de este parámetro (Tabla 13). Sin embargo, este cambio se atenuó al final del período de refrigeración a 8 ºC. No se observaron diferencias significativas de a^*, tras la aplicación de los tratamientos, mientras que sus valores disminuyeron en las muestras tratadas con presión aplicada individualmente o en combinación, al final del almacenamiento. Por otro lado, los antimicrobianos aplicados individualmente no afectaron a los valores de a^*. En cuanto a la tendencia al amarillo, no se observaron diferencias significativas de b^* tras la aplicación de los tratamientos. Sin embargo, a los 60 d de refrigeración a 8 ºC se observó un aumento (P<0.05) de este parámetro en el jamón tratado con LF y una disminución (P<0.05) en el presurizado y combinado con el SLP. Según los distintos trabajos publicados (Martínez *et al*., 1988; Ridley y Shalo, 1990), el SLP ejerció un efecto leve sobre las características físicas y sensoriales de la leche y de los productos lácteos. Sin embargo, en salmón ahumado, Montiel *et al*. (2012) observaron que los valores de L^*, a^* y b^* resultaron afectados por las altas presiones y el SLP, registrándose un aumento ligero de L^* con los tratamientos, mientras que el impacto sobre a^* y b^* fue bajo. Se han publicado pocos estudios sobre el efecto de la LF y del SLP en productos cárnicos. Tan y Ockerman (2008) evaluaron el efecto del SLP sobre las características físicas y sensoriales de muslos de pollo marinados. Estos autores observaron una reducción de a^* en las muestras tratadas con el SLP que atribuyeron a la adición de peróxido de hidrógeno (H_2O_2), agente oxidante usado ocasionalmente como blanqueante en la industria alimentaria (Daeschel y Penner, 1992). En el presente trabajo, los parámetros del color no fueron afectados por el SLP tras el tratamiento, demostrando que la generación *in situ* de H_2O_2 por la glucosa oxidasa (GOX) y la glucosa añadidas no afectaría negativamente al color del jamón curado.

Tabla 12. Parámetros de color del jamón curado tratado con altas presiones a 400 y 500 MPa durante 10 min, nisina, pediocina y sus combinaciones, mantenido durante 60 d a 8 ºC.

		1 d	30 d	60 d
*L**	Control	44.73± 4.41^{cB}	40.53±4.61^{abcA}	38.58±5.17^{abA}
	Nisina	42.13±3.21^{abcB}	42.45±4.70^{bcdB}	38.29±4.15^{aA}
	Pediocina	41.75±3.66^{abB}	39.26±3.25^{aA}	39.57±4.42^{abA}
	400 MPa	42.66±3.30^{abcB}	39.81±5.33^{abA}	39.93±4.47^{abcA}
	500 MPa	44.03±4.55^{bcA}	44.22±4.10^{dA}	43.11±5.20^{cdA}
	400 MPa + Nis	44.19±5.24^{bcB}	43.12±3.85^{cdAB}	41.31±3.92^{abcdA}
	400 MPa + Ped	44.59±5.04^{cB}	42.16±3.41^{bcdA}	44.35±6.12^{dAB}
	500 MPa + Nis	40.48±4.10^{aA}	44.54±5.13^{dB}	42.81±4.20^{cdB}
	500 MPa + Ped	40.83±5.43^{aA}	42.39±3.26^{bcdA}	41.49±5.20^{bcdA}
*a**	Control	19.41±2.48^{abA}	21.02±2.55^{cB}	19.67±2.55^{eA}
	Nisina	21.41±1.86^{dC}	20.18±2.07^{cB}	18.83±3.00^{cdeA}
	Pediocina	19.92±2.18^{bcdAB}	20.45±2.84^{cB}	18.59±2.92^{cdeA}
	400 MPa	18.16±2.19^{aA}	20.08±2.10^{bcB}	16.99±3.83^{bcA}
	500 MPa	19.37±1.77^{abC}	18.04±2.39^{aB}	16.30±3.53^{abA}
	400 MPa + Nis	19.77±2.38^{abcA}	19.85±2.23^{bcA}	19.04±2.24^{deA}
	400 MPa + Ped	21.21±2.26^{cdC}	19.71±1.52^{abcB}	17.46±2.83^{bcdA}
	500 MPa + Nis	19.62±2.11^{abcB}	20.00±2.31^{bcB}	18.04±2.26^{bcdeA}
	500 MPa + Ped	20.82±2.29^{bcdC}	18.42±2.34^{abB}	14.98±1.92^{aA}
*b**	Control	19.75±3.32^{bcB}	20.05±4.56^{abB}	17.85±3.34^{bA}
	Nisina	20.18±3.14^{cB}	19.52±3.38^{abAB}	17.55±3.28^{bA}
	Pediocina	18.71±2.52^{abcA}	18.31±4.42^{aA}	17.49±2.66^{bA}
	400 MPa	17.87±3.55^{abAB}	19.31±3.17^{abB}	16.75±2.78^{abA}
	500 MPa	19.00±4.33^{abcA}	19.33±3.61^{abA}	19.13±4.78^{bA}
	400 MPa + Nis	20.04±2.94^{bcAB}	20.40±2.83^{abB}	18.53±2.92^{bA}
	400 MPa + Ped	22.40±4.00^{dC}	19.75±2.56^{abB}	17.85±3.41^{bA}
	500 MPa + Nis	16.92±3.52^{aA}	21.29±4.50^{bB}	18.68±5.15^{bA}
	500 MPa + Ped	19.81±3.29^{bcB}	19.37±3.40^{abB}	14.84±4.21^{aA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 13. Parámetros de color del jamón curado tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF y sus combinaciones, mantenido durante 60 d a 8 ºC.

		1 d	30 d	60 d
*L**	Control	40.05 ± 2.44 ^{abB}	38.78 ± 2.58 ^{abAB}	37.65 ± 3.29 ^{abA}
	SLP	39.32 ± 2.71 ^{aA}	39.60 ± 3.37 ^{abA}	38.05 ± 3.40 ^{abcA}
	LF	39.64 ± 3.15 ^{abB}	37.76 ± 3.67 ^{aA}	37.24 ± 3.07 ^{aA}
	450 MPa	40.38 ± 3.60 ^{abA}	40.77 ± 3.14 ^{bcA}	39.78 ± 3.23 ^{bcA}
	450 MPa + SLP	42.28 ± 2.30 ^{cB}	40.19 ± 3.84 ^{bcA}	39.69 ± 4.20 ^{bcA}
	450 MPa + LF	41.38 ± 2.53 ^{bcAB}	41.86 ± 1.82 ^{cB}	40.25 ± 3.39 ^{cA}
*a**	Control	14.57 ± 1.71 ^{aB}	13.24 ± 2.80 ^{aA}	15.42 ± 2.66 ^{cB}
	SLP	14.61 ± 1.48 ^{aA}	14.67 ± 2.03 ^{bA}	14.67 ± 3.15 ^{bcA}
	LF	14.87 ± 1.81 ^{aA}	14.12 ± 1.81 ^{abA}	15.85 ± 2.13 ^{cB}
	450 MPa	14.24 ± 1.71 ^{aB}	14.36 ± 2.27 ^{bB}	13.07 ± 2.65 ^{aA}
	450 MPa + SLP	14.70 ± 1.85 ^{aB}	15.00 ± 2.27 ^{bB}	13.30 ± 2.05 ^{abA}
	450 MPa + LF	15.09 ± 0.98 ^{aB}	14.98 ± 1.16 ^{bB}	13.73 ± 2.05 ^{abA}
*b**	Control	8.85 ± 1.50 ^{abA}	12.72 ± 3.19 ^{bB}	8.63 ± 3.31 ^{abA}
	SLP	8.51 ± 2.11 ^{abA}	14.74 ± 2.53 ^{cB}	7.95 ± 3.02 ^{aA}
	LF	8.18 ± 1.81 ^{aA}	13.18 ± 2.69 ^{bcC}	9.83 ± 2.63 ^{bB}
	450 MPa	8.12 ± 1.67 ^{aA}	10.45 ± 2.75 ^{aB}	7.91 ± 3.31 ^{aA}
	450 MPa + SLP	9.15 ± 1.57 ^{abB}	10.29 ± 2.57 ^{aB}	7.61 ± 2.57 ^{aA}
	450 MPa + LF	9.45 ± 1.52 ^{bA}	10.92 ± 1.55 ^{aB}	8.32 ± 3.05 ^{abA}

Los valores son la media ±SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

En relación a la textura del jamón curado (Tabla 14), la presurización redujo la resistencia al corte en las muestras de jamón curado presurizado a 500 o 600 MPa durante 5 min comparadas con las muestras no presurizadas y las sometidas a tratamientos más suaves de 400 MPa. Respecto a la fuerza máxima, las diferencias fueron significativas ($P<0.05$) solo en las muestras presurizadas al final del período de almacenamiento a 8 ºC. El tiempo de almacenamiento en refrigeración afectó ($P<0.001$) a ambas propiedades de textura, detectándose valores menores al final del almacenamiento, tanto en el jamón no presurizado como en el tratado por altas presiones (De Alba *et al*., 2012a).

Tabla 14. Propiedades de textura del jamón curado tratado con altas presiones a 400, 500 y 600 MPa durante 5 min, mantenido durante 60 d a 8 ºC.

		Control	400 MPa/5 min	500 MPa/5 min	600 MPa/5 min
Fuerza al corte (N)	1 d	36.09 ± 2.86^{bBC}	34.49 ± 9.31^{bBC}	23.74 ± 5.75^{aAB}	20.96 ± 9.82^{aA}
	15 d	42.37 ± 14.62^{bCD}	31.89 ± 6.16^{abB}	25.51 ± 4.26^{aB}	20.60 ± 3.83^{aA}
	30 d	54.96 ± 9.57^{cD}	43.16 ± 11.41^{bC}	35.05 ± 7.20^{aC}	32.49 ± 12.85^{aB}
	45 d	27.40 ± 3.28^{bAB}	27.58 ± 2.16^{bB}	24.13 ± 2.36^{abAB}	20.34 ± 2.62^{aA}
	60 d	17.38 ± 2.33^{aA}	16.81 ± 1.32^{aA}	20.77 ± 1.02^{bA}	18.89 ± 1.95^{abA}
Fuerza máxima (N)	1 d	338.90 ± 50.79^{aA}	351.68 ± 97.26^{aA}	341.21 ± 31.65^{aA}	306.74 ± 54.96^{aA}
	15 d	319.13 ± 50.14^{aA}	314.61 ± 62.90^{aA}	397.27 ± 116.68^{aA}	341.48 ± 36.26^{aA}
	30 d	330.47 ± 62.12^{aA}	331.39 ± 37.44^{aA}	385.05 ± 123.71^{aA}	395.19 ± 75.07^{aA}
	45 d	304.56 ± 56.01^{aA}	361.45 ± 54.31^{aA}	357.47 ± 66.09^{aA}	373.22 ± 73.77^{aA}
	60 d	268.34 ± 35.21^{aA}	331.25 ± 57.94^{bA}	318.01 ± 35.13^{abA}	370.65 ± 78.80^{bA}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

En jamón presurizado a 500 MPa durante 10 min, o con pediocina o nisina aplicadas individualmente y como se muestra en la Tabla 15, se registraron valores significativamente ($P<0.05$) menores de la fuerza al corte, 1 d después de los tratamientos. Los valores de este parámetro tendían a aumentar durante la refrigeración, aunque las variaciones no mostraron una tendencia clara. Respecto a la fuerza máxima, se detectaron valores mayores ($P<0.05$) en las muestras tratadas con nisina o con la combinación de 400 MPa y nisina o pediocina, sin una tendencia clara durante el período de refrigeración (De Alba *et al*., 2013a).

Tabla 15. Parámetros de textura del jamón curado tratado con altas presiones a 400 y 500 MPa durante 10 min, nisina, pediocina y sus combinaciones, mantenido durante 60 d a 8 ºC.

		1 d	30 d	60 d
Fuerza al corte (N)	Control	35.62±11.95^{cA}	32.48±5.18^{bcA}	38.37±9.42^{dA}
	Nisina	27.05±11.11^{abA}	34.12±3.57^{abB}	38.16±10.10^{dB}
	Pediocina	25.17±7.96^{aA}	41.90±3.33^{eB}	30.74±12.76^{bcA}
	400 MPa	37.13±11.77^{cA}	34.11±5.40^{abA}	34.32±9.96^{cdA}
	500 MPa	27.31±8.26^{abA}	36.35±14.65^{bcdB}	30.17±6.94^{abcA}
	400 MPa + Nis	31.81±7.68^{abcB}	39.04±7.33^{cdeC}	26.30±5.51^{abA}
	400 MPa + Ped	30.92±8.85^{abcA}	40.12±4.33^{deB}	29.10±8.42^{abcA}
	500 MPa + Nis	32.45±13.64^{bcB}	36.19±12.47^{bcdB}	23.05±5.69^{aA}
	500 MPa + Ped	33.35±6.42^{bcB}	30.91±8.05^{aAB}	28.87±8.02^{abcA}
Dureza (N)	Control	336.78±133.84^{aA}	338.67±35.40^{abA}	393.76±46.29^{aA}
	Nisina	421.51±116.39^{bcdB}	386.44±62.46^{bcA}	362.64±51.10^{aA}
	Pediocina	345.66±97.11^{aA}	311.71±59.06^{aA}	396.45±45.56^{aA}
	400 MPa	353.62±14.96^{aAB}	355.99±50.13^{abcA}	400.06±71.23^{aB}
	500 MPa	391.44±99.88^{abcA}	533.40±54.01^{eB}	387.16±57.85^{aA}
	400 MPa + Nis	450.35±78.66^{cdA}	408.76±70.34^{cdA}	511.38±106.67^{bA}
	400 MPa + Ped	467.27±63.79^{dB}	453.15±81.99^{dAB}	425.47±49.23^{aA}
	500 MPa + Nis	398.32±77.25^{abcA}	552.48±98.97^{eA}	413.52±96.10^{aA}
	500 MPa + Ped	377.08±120.92^{abA}	389.29±50.20^{bcA}	413.45±95.09^{aA}

Los valores son la media ±SD. Las medias dentro de la misma columna con diferente superíndice en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferente superíndice en mayúscula difieren significativamente a $P<0.05$.

La alta presión induce alteración de la estructura de la carne y de la distribución del agua, afectando a la jugosidad y a la terneza en función de la composición del producto, del nivel de presión y de la combinación presión/temperatura. En este sentido, Bertram *et al*. (2006) observaron que la presurización afectaba a la estructura de la carne y la distribución del agua del jamón cocido, a la vez que mejoraba su jugosidad y aumentaba la terneza. Sin embargo, la información publicada sobre el efecto de los tratamientos de altas presiones en la textura del

jamón curado determinada instrumentalmente es escasa. Serra *et al*. (2007) observaron un aumento de fibrosidad pero no de dureza en jamones presurizados a 600 MPa, sin afectar negativamente a la calidad sensorial. Otros autores (Clariana *et al*. 2011; Fuentes *et al*., 2010; Fulladosa *et al*., 2009) detectaron un aumento de la dureza, gomosidad y fibrosidad del jamón curado presurizado a 600 MPa. Estos cambios podrían ser originados por la desnaturalización parcial de las proteínas musculares y su modificación, como resultado de la oxidación proteica (Fuentes *et al*., 2010). También Clariana *et al*. (2011) registraron un aumento de la masticabilidad, de la intensidad del aroma y de la salinidad del jamón curado presurizado mantenido en condiciones de refrigeración durante 50 d. Sin embargo, el tratamiento a 450 MPa durante 10 min no afectó a las características sensoriales del jamón Serrano loncheado almacenado a 4 ºC (Morales *et al*., 2006).

En el jamón tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF o sus combinaciones no se observaron diferencias significativas en los valores de la fuerza al corte ni de la fuerza máxima (Tabla 16) tras los tratamientos, excepto en el jamón tratado con LF, en el que se registró un aumento (P<0.05) de la fuerza máxima.

Tabla 16. Propiedades de textura del jamón curado tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF y sus combinaciones, mantenido durante 60 d a 8 ºC.

		1 d	30 d	60 d
Fuerza al corte (N)	Control	38.23 ± 10.56^{aA}	35.56 ± 10.82^{abA}	37.38 ± 9.09^{aA}
	SLP	35.70 ± 8.78^{aA}	30.56 ± 4.29^{aA}	32.71 ± 7.77^{aA}
	LF	36.99 ± 12.71^{aA}	36.53 ± 4.52^{abA}	37.21 ± 15.83^{aA}
	450 MPa	36.05 ± 6.90^{aA}	37.93 ± 6.00^{bA}	38.93 ± 10.25^{aA}
	450 MPa + SLP	30.14 ± 6.82^{aA}	36.73 ± 11.20^{abAB}	38.06 ± 4.76^{aB}
	450 MPa + LF	38.11 ± 16.12^{aA}	34.75 ± 14.28^{abA}	38.91 ± 12.83^{aA}
Dureza (N)	Control	405.88 ± 59.59^{aA}	428.80 ± 64.47^{aA}	441.22 ± 112.81^{aA}
	SLP	481.01 ± 47.56^{abA}	504.37 ± 163.14^{aA}	444.77 ± 113.26^{aA}
	LF	542.33 ± 91.76^{bB}	468.19 ± 85.04^{aAB}	374.50 ± 124.99^{aA}
	450 MPa	438.98 ± 155.72^{abA}	445.31 ± 60.48^{aA}	467.83 ± 136.76^{aA}
	450 MPa + SLP	458.09 ± 95.63^{abA}	504.76 ± 98.58^{aA}	453.68 ± 104.41^{aA}
	450 MPa + LF	467.23 ± 105.96^{abB}	449.80 ± 50.50^{aB}	348.86 ± 133.20^{aA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferente superíndice en minúscula difieren significativamente a P<0.05. Las medias dentro de la misma fila con diferente superíndice en mayúscula difieren significativamente a P<0.05.

Después de 60 d en refrigeración a 8 ºC, la fuerza máxima disminuyó (P<0.05) en el jamón tratado con LF o en combinación con el tratamiento de alta presión y la fuerza

al corte experimentó un aumento (P<0.05) con el SLP combinado con la presurización. No obstante, al final del período investigado no se registraron diferencias significativas entre el jamón sometido a los diferentes tratamientos y el control en ninguna de las propiedades de textura estudiadas. En salmón ahumado, los valores de dureza y de la fuerza al corte tendían a aumentar a 450 MPa o en combinación con el SLP (Montiel *et al*., 2012), mientras que la LF no afectó a las características de albóndigas (Colak *et al*., 2008).

4.2. TRATAMIENTOS EN CARPACCIO DE TERNERA

4.2.1. TRATAMIENTOS COMBINADOS EN LA INACTIVACIÓN DE *L. monocytogenes*, *S.* Enteritidis y *E. coli* EN CARPACCIO DE TERNERA

Con el objetivo de inactivar *S*. Enteritidis en carpaccio de ternera y minimizar los cambios indeseables que pudiera originar la presurización en este producto cárnico, se redujo la intensidad de los tratamientos aplicando altas presiones a 450 MPa durante 5, 10 o 15 min. Los valores de letalidad para *Salmonella* en carpaccio fueron superiores a los alcanzados en jamón curado (De Alba *et al*., 2012a), hecho atribuible a la diferencia en a_w de ambos productos, mayor en carpaccio y por tanto, con menor efecto baroprotector (Patterson, 2005). Como se muestra en la Tabla 17, inmediatamente después de la aplicación de 450 MPa durante 5 y 10 min se alcanzaron reducciones de 3.68 y 5.94 unidades logarítmicas, mientras que *Salmonella* solo se detectó por enriquecimiento de las muestras tratadas a 450 MPa durante 15 min. Los recuentos del patógeno disminuyeron únicamente 0.26 unidades logarítmicas en las muestras no presurizadas tras los 30 d de almacenamiento a 8 ºC, indicando la capacidad de *Salmonella* de sobrevivir en este producto cárnico. Durante la refrigeración, las reducciones con el tratamiento menos intenso fueron de 1.33 unidades logarítmicas y la presencia de *Salmonella* en 10 g de producto solo fue detectada tras el enriquecimiento de las muestras con los tratamientos de presurización más intensos, indicando que el patógeno no fue capaz de recuperarse bajo las condiciones ensayadas.

Tabla 17. Niveles (log ufc/g) de *S.* Enteritidis en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5, 10 y 15 min, mantenido durante 30 d a 8 ºC.

	Control	450 MPa/5min	450 MPa/10min	450 MPa/15min
0 h	6.54 ± 0.08^{dCD}	2.86 ± 0.07^{cE}	0.60 ± 0.35^{bA}	PRE^{aA}
1 d	6.41 ± 0.03^{cB}	1.93 ± 0.15^{bD}	PRE^{aA}	PRE^{aA}
3 d	6.45 ± 0.08^{cBC}	3.44 ± 0.09^{bF}	PRE^{aA}	PRE^{aA}
7 d	6.55 ± 0.05^{cD}	2.00 ± 0.00^{bD}	PRE^{aA}	PRE^{aA}
15 d	6.45 ± 0.03^{cBCD}	1.45 ± 0.63^{bB}	PRE^{aA}	PRE^{aA}
21 d	6.29 ± 0.10^{cA}	1.08 ± 0.90^{bA}	PRE^{aA}	PRE^{aA}
30 d	6.28 ± 0.03^{cA}	1.53 ± 0.35^{bC}	PRE^{aA}	PRE^{aA}

PRE, presencia en 10 g. Límite de detección de 10 ufc/g.
Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Respecto a los microorganismos viables totales, se comprobó en el carpaccio control un aumento en los niveles (Tabla 18) durante el almacenamiento a 8 ºC, mientras que la presurización mantuvo los recuentos en valores significativamente menores ($P<0.05$) que los detectados en el carpaccio control. Se han publicado pocos trabajos de presurización de carpaccio. En carpaccio de cerdo, Realini *et al*. (2011) observaron la reducción de los niveles de viables totales en muestras tratadas a 400 y 600 MPa. Por otro lado, Garriga *et al*. (2004) también detectaron reducciones de al menos 4 unidades logarítmicas en el contenido de viables totales en lomo de ternera marinado después de la aplicación de 600 MPa durante 6 min.

Tabla 18. Niveles (log ufc/g) de viables totales en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5, 10 y 15 min, mantenido durante 30 d a 8 ºC.

	Control	450 MPa /5 min	450 MPa /10 min	450 MPa /15 min
0 h	6.10 ± 0.79^{bA}	3.56 ± 0.38^{abA}	2.98 ± 2.04^{aA}	2.67 ± 1.79^{aA}
15 d	7.10 ± 0.47^{dB}	5.70 ± 1.17^{bB}	6.55 ± 0.37^{cB}	4.65 ± 0.10^{aA}
30 d	7.06 ± 0.23^{dB}	5.73 ± 0.14^{bB}	6.38 ± 0.02^{cB}	4.39 ± 0.13^{aA}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Se seleccionó el tratamiento de 450 MPa durante 5 min para los ensayos posteriores realizados en carpaccio de ternera, al ocasionar cambios mínimos de color, como se recoge en los apartados correspondientes a las características del producto, y reducir el patógeno hasta niveles que permitirían estudiar posibles sinergias entre los tratamientos combinados.

Con objeto de inactivar *L. monocytogenes*, *S*. Enteritidis y *E. coli* O157:H7 y observar el comportamiento de los patógenos en condiciones de abuso de temperatura a 8 ºC y a temperatura ambiente de 22 ºC durante 7 d, se aplicaron tratamientos individuales de 450 MPa durante 5 min, el SLP, LF activada (ALF), y sus combinaciones. La temperatura ambiente de 22 ºC fue elegida para simular interrupción de la cadena de frío. Según las Tablas 19 a 24, *L. monocytogenes*, *S*. Enteritidis y *E. coli* O157:H7 no fueron capaces de crecer en el carpaccio no tratado durante el período investigado, ni siquiera a 22 ºC, apuntando a la actividad antimicrobiana de los agentes del curado incluso en condiciones de interrupción de la cadena de frío. Los niveles de *S*. Enteritidis disminuyeron durante el período de almacenamiento investigado.

Las altas presiones ejercieron un efecto bactericida sobre los tres patógenos, aunque *S*. Enteritidis mostró la mayor sensibilidad al tratamiento de presurización, seguido de *E. coli* y *L. monocytogenes*. En general, las bacterias Gram-negativas son más sensibles a las altas presiones que las Gram-positivas, aunque la inactivación bacteriana es altamente variable, tanto entre microorganismos pertenecientes al mismo tipo (Garriga *et al*., 2002) como entre cepas de la misma especie (Alpas *et al*., 1999). Las reducciones de *S*. Enteritidis fueron superiores a 5 unidades logarítmicas en carpaccio de ternera presurizado a 450 MPa durante 5 min después de 30 d a 8 ºC (De Alba *et al*., 2012b).

No se detectó actividad antimicrobiana del SLP ni de la ALF, aplicados individualmente en el carpaccio de ternera (Tablas 19 a 24). Al igual que en jamón curado loncheado, esta ausencia de actividad podría estar relacionada con la aplicación irregular de los antimicrobianos en las muestras, la interferencia con componentes de la carne o con agentes del curado presentes en el carpaccio o con el tamaño del inóculo.

La combinación de altas presiones y el SLP produjo un efecto bactericida sinérgico frente a *S*. Enteritidis y *E. coli* O157:H7 en carpaccio, tras la aplicación de los tratamientos y a los 7 d de almacenamiento a 8 y 22 ºC. En el caso de *L. monocytogenes* y *E. coli* O157:H7, las reducciones detectadas a los 7 d de almacenamiento en las muestras tratadas con el SLP en combinación con altas presiones fueron mayores a 22 ºC que a 8 ºC, al contrario de lo que ocurrió con *Salmonella*. La actividad antimicrobiana del SLP está relacionada con la disrupción de las actividades metabólicas celulares, por lo que su efecto podría reducirse a temperaturas más bajas en que las células no sean metabólicamente activas. El tratamiento combinado entre ALF y altas presiones no aportó ninguna mejora al

potencial antimicrobiano de las altas presiones frente a los tres patógenos estudiados (Tablas 19 a 24). En filetes de pollo, Del Olmo *et al*. (2012b) no detectaron sinergia frente a *E. coli* O157:H7, aunque sí sobre *P. fluorescens*. Sin embargo, el efecto bactericida del tratamiento combinado sobre *L. monocytogenes* fue leve (Del Olmo *et al*. 2012a).

Tabla 19. Niveles (log ufc/g) de *L. monocytogenes* en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 7 d a 8 ºC.

	0 h	24 h	48 h	7d
Control	6.67 ± 0.12^{cBC}	6.58 ± 0.09^{bAB}	6.70 ± 0.08^{cB}	6.48 ± 0.14^{cA}
SLP	6.62 ± 0.15^{cA}	6.53 ± 0.19^{bA}	6.57 ± 0.10^{cA}	6.42 ± 0.10^{cA}
ALF	6.64 ± 0.16^{cA}	6.65 ± 0.05^{bA}	6.49 ± 0.09^{cA}	6.52 ± 0.05^{cA}
450 MPa	5.44 ± 0.17^{bC}	4.82 ± 0.56^{aA}	5.15 ± 0.10^{bB}	4.94 ± 0.42^{bA}
450 MPa + SLP	4.86 ± 0.08^{aB}	4.68 ± 0.50^{aB}	4.91 ± 0.18^{aB}	4.28 ± 0.13^{aA}
450 MPa + ALF	5.51 ± 0.12^{bC}	5.12 ± 1.82^{abB}	5.19 ± 0.16^{bB}	4.82 ± 0.27^{bA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 20. Niveles (log ufc/g) de *L. monocytogenes* en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 7 d a 22 ºC.

	0 h	24 h	48 h	7d
Control	6.62 ± 0.37^{cA}	6.51 ± 0.30^{cA}	6.48 ± 0.34^{cA}	6.40 ± 0.50^{cA}
SLP	6.65 ± 0.23^{cB}	6.56 ± 0.30^{cB}	6.30 ± 0.56^{cA}	6.40 ± 0.49^{cA}
ALF	6.52 ± 0.36^{bA}	6.38 ± 0.38^{cA}	6.41 ± 0.45^{cA}	6.43 ± 0.45^{cA}
450 MPa	5.65 ± 0.77^{aD}	5.19 ± 0.74^{aC}	4.64 ± 0.62^{aB}	4.18 ± 1.46^{bA}
450 MPa + SLP	5.60 ± 0.62^{aC}	5.13 ± 0.97^{aB}	5.07 ± 1.04^{bAB}	3.73 ± 0.48^{aA}
450 MPa + ALF	5.69 ± 0.66^{aB}	5.43 ± 0.91^{bB}	5.23 ± 1.13^{bAB}	4.81 ± 0.86^{bA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 21. Niveles (log ufc/g) de *S.* Enteritidis en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 7 d a 8 ºC.

	0 h	24 h	48 h	7d
Control	5.83 ± 0.21^{bB}	5.72 ± 0.09^{dB}	4.97 ± 0.19^{cA}	4.93 ± 0.47^{deA}
SLP	5.67 ± 0.07^{bC}	5.26 ± 0.26^{dBC}	5.02 ± 0.17^{cAB}	4.83 ± 0.36^{dA}
ALF	5.83 ± 0.21^{bC}	5.37 ± 0.14^{dB}	4.87 ± 0.18^{cA}	5.14 ± 0.13^{eA}
450 MPa	3.18 ± 0.13^{aB}	2.39 ± 0.29^{bAB}	1.08 ± 0.66^{bA}	2.13 ± 0.87^{cAB}
450 MPa + SLP	2.35 ± 1.26^{aA}	0.56 ± 0.08^{aA}	0.15 ± 0.21^{aA}	0.39 ± 0.83^{aA}
450 MPa + ALF	3.09 ± 0.17^{aB}	3.08 ± 0.33^{cB}	1.44 ± 0.24^{bA}	1.75 ± 0.80^{bA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 22. Niveles (log ufc/g) de *S.* Enteritidis en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 7 d a 22 ºC.

	0 h	24 h	48 h	7d
Control	6.04 ± 0.15^{bC}	5.78 ± 0.45^{cA}	5.60 ± 0.05^{dBC}	5.16 ± 0.50^{cA}
SLP	5.99 ± 0.18^{bC}	5.55 ± 0.31^{cB}	5.51 ± 1.22^{cB}	5.02 ± 0.51^{cA}
ALF	6.13 ± 0.22^{bC}	5.61 ± 0.58^{cB}	5.71 ± 1.49^{cB}	5.04 ± 0.65^{cA}
450 MPa	3.40 ± 0.24^{aB}	2.73 ± 0.13^{abB}	2.03 ± 0.49^{bA}	1.49 ± 0.83^{bA}
450 MPa + SLP	2.35 ± 1.41^{aA}	1.83 ± 1.51^{aA}	1.88 ± 1.74^{aA}	0.65 ± 0.92^{aA}
450 MPa + ALF	3.44 ± 1.80^{aA}	2.41 ± 0.20^{bA}	2.12 ± 0.36^{bA}	1.28 ± 0.28^{abA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferente superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 23. Niveles (log ufc/g) de *E. coli* O157:H7 en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 7 d a 8 ºC.

	0 h	24 h	48 h	7d
Control	6.68 ± 0.07^{dA}	6.62 ± 0.08^{dA}	6.75 ± 0.06^{dA}	6.66 ± 0.05^{cA}
SLP	6.63 ± 0.29^{dA}	6.72 ± 0.17^{dAB}	6.81 ± 0.04^{dB}	6.58 ± 0.14^{cA}
ALF	6.69 ± 0.08^{dA}	6.78 ± 0.04^{dA}	6.71 ± 0.07^{dA}	6.73 ± 0.08^{cA}
450 MPa	5.02 ± 0.14^{cB}	4.92 ± 0.21^{cAB}	4.86 ± 0.29^{cB}	4.63 ± 0.41^{bA}
450 MPa + SLP	4.22 ± 1.06^{aB}	1.74 ± 2.00^{aA}	4.45 ± 0.54^{aBC}	4.64 ± 0.15^{bC}
450 MPa + ALF	4.48 ± 0.11^{bAB}	4.37 ± 0.56^{bA}	4.70 ± 0.40^{bB}	4.29 ± 0.48^{aA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferente superíndice en mayúscula difieren significativamente a $P<0.05$.

Tabla 24. Niveles (log ufc/g) de *E. coli* O157:H7 en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 7 d a 22 ºC.

	0 h	24 h	48 h	7d
Control	6.24 ± 0.12^{cA}	6.42 ± 0.17^{dC}	6.32 ± 0.24^{deAB}	6.36 ± 0.06^{deBC}
SLP	6.40 ± 0.10^{cdAB}	6.34 ± 0.07^{dA}	6.45 ± 0.13^{eAB}	6.48 ± 0.05^{eB}
ALF	6.50 ± 0.12^{dC}	6.38 ± 0.14^{dBC}	6.28 ± 0.09^{dAB}	6.20 ± 0.05^{dA}
450 MPa	4.72 ± 0.57^{aC}	3.89 ± 0.35^{cB}	3.89 ± 0.88^{cB}	3.47 ± 0.46^{cA}
450 MPa + SLP	4.74 ± 0.06^{aA}	0.00 ± 0.00^{aA}	1.00 ± 1.15^{aA}	1.52 ± 1.76^{aA}
450 MPa + ALF	5.06 ± 0.07^{bC}	3.62 ± 0.07^{bAB}	3.62 ± 0.77^{bB}	3.27 ± 0.88^{bA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

En las muestras de carpaccio tratadas con el SLP, ALF o altas presiones a 450 MPa durante 5 min (Tabla 25), los niveles de microorganismos viables totales se redujeron 3.28, 3.24, y 3.55 unidades logarítmicas, respectivamente, tras la aplicación de los tratamientos. Con la combinación de la presurización y ALF o el SLP, las reducciones registradas fueron de 3.69 y 3.82 unidades logarítmicas. Aunque durante el almacenamiento a 8 ºC, los niveles de microorganismos viables totales aumentaron en el carpaccio control y en el tratado, se mantuvieron en valores más bajos en las muestras tratadas hasta el final del período investigado.

Tabla 25. Niveles (log ufc/g) de viables totales en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 30 d a 8 ºC.

	0 h	1 d	7 d	15 d	30 d
Control	6.81 ± 0.34^{dA}	7.05 ± 0.30^{eB}	7.64 ± 0.24^{eC}	7.74 ± 0.25^{dC}	8.27 ± 0.12^{eD}
SLP	3.53 ± 0.19^{cA}	3.78 ± 0.09^{dB}	3.73 ± 0.03^{dB}	3.46 ± 0.09^{bA}	5.14 ± 0.15^{bC}
ALF	3.57 ± 0.03^{cAB}	3.52 ± 0.18^{cA}	3.66 ± 0.07^{dB}	3.60 ± 0.09^{bAB}	5.41 ± 0.02^{cC}
450 MPa	3.26 ± 0.22^{bB}	3.11 ± 0.09^{bA}	3.44 ± 0.13^{cC}	4.17 ± 0.50^{cD}	6.29 ± 0.12^{dE}
450 MPa + SLP	2.99 ± 0.14^{aB}	2.95 ± 0.09^{aB}	2.88 ± 0.12^{aAB}	2.75 ± 0.20^{aA}	4.52 ± 0.07^{aC}
450 MPa + ALF	3.12 ± 0.09^{abA}	3.15 ± 0.04^{bA}	3.10 ± 0.10^{bA}	3.33 ± 0.48^{bA}	4.54 ± 0.22^{aB}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

La siguiente combinación ensayada en este producto frente a *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 consistió en aplicar tratamientos de 450 MPa durante 5 min con las bacteriocinas enterocinas A y B, pediocina PA-1, nisina Z, y nisina y pediocina comerciales. Las bacteriocinas aplicadas individualmente produjeron reducciones entre 0.1 y 1.5 unidades logarítmicas en los recuentos de *L.*

monocytogenes (Tabla 26), siendo la pediocina comercial la bacteriocina más efectiva frente al patógeno. Por otro lado, la nisina Z fue la bacteriocina menos efectiva frente a *Listeria*. El mecanismo de resistencia a la nisina parece estar correlacionado con cambios en la composición de los ácidos grasos de la membrana, con la estructura de la pared celular y con requerimientos de cationes divalentes (Crandall y Montville, 1998). En el caso de *S*. Enteritidis y *E. coli* O157:H7 (Tabla 27 y 28), las bacteriocinas aplicadas individualmente no mostraron actividad antimicrobiana.

El tratamiento de presurización de 450 MPa durante 5 min fue más efectivo frente a *S*. Enteritidis, con reducciones superiores a 4 unidades logarítmicas, seguido de *E. coli* O157:H7 y *L. monocytogenes*, que mostraron mayor barotolerancia, como se había observado en los estudios anteriores. Inmediatamente después de la presurización se detectó un efecto antimicrobiano ligeramente sinérgico frente a *Listeria* con los tratamientos combinados de enterocinas A y B o nisina comercial con las altas presiones. Sin embargo, con la combinación de pediocina PA-1, nisina Z o pediocina comercial el efecto antimicrobiano solo fue aditivo (Tabla 26). En el caso de *S*. Enteritidis (Tabla 27), el tratamiento combinado entre las bacteriocinas y las altas presiones no aportó ninguna mejora al potencial antimicrobiano de la presurización. En el caso de *E. coli* (Tabla 28), se observó un efecto antimicrobiano ligeramente sinérgico con la mayoría de los tratamientos combinados, excepto con la pediocina PA-1 con la que se registró un efecto aditivo. Al final del período de almacenamiento, el efecto antimicrobiano sobre *Listeria* fue aditivo (Tabla 26) con la combinación de las enterocinas A y B o la nisina comercial y las altas presiones, y ligeramente sinérgico con la nisina Z o la pediocina comercial con la presurización. Garriga *et al*. (2002) observaron que tratamientos combinados de alta presión a 400 MPa durante 10 min y sakacina K, enterocinas A y B o pediocina AcH conseguían mantener a *L. monocytogenes* por debajo del límite de detección en jamón cocido loncheado durante 61 d a 4 ºC. En el caso de *Salmonella* (Tabla 27), se detectó un efecto antimicrobiano aditivo entre las altas presiones y las enterocinas A y B o la pediocina PA-1. En jamón curado, Jofré *et al*. (2008) detectaron que la combinación de 600 MPa durante 5 min y enterocinas A y B, sakacina K o nisina producía ausencia de *Salmonella*. En el caso de *E. coli* (Tabla 28), se observó un efecto antimicrobiano sinérgico con los tratamientos combinados entre las altas presiones y las enterocinas A y B, la pediocina PA-1 o la nisina comercial, detectándose índices de inactivación 2 unidades logarítmicas superiores a los alcanzados con la suma de los tratamientos individuales. Este efecto fue menor con *E. coli* O157:H7 en jamón curado loncheado con la combinación de nisina y 500 MPa durante 10 min (De Alba *et al*., 2013a), confirmando de nuevo el efecto baroprotector de la a_w baja sobre el patógeno. En general, los resultados obtenidos indican que cada bacteriocina puede mostrar

efectos diferentes en combinación con las altas presiones, dependiendo de la célula diana. Durante el almacenamiento a 8 ºC, ninguno de los tres patógenos estudiados creció en carpaccio no tratado (Tablas 26 a 28).

Las condiciones del producto, con sales del curado y una a_w con valores comprendidos entre 0.916-0.926, ejercieron un efecto bacteriostático sobre *E. coli* y ligeramente bactericida sobre *Listeria* y *Salmonella*. *E. coli* es capaz de sobrevivir en otros productos cárnicos como el jamón curado (De Alba *et al*., 2013a) y en embutidos fermentados (Holck *et al*., 2011). Se ha descrito (Rhoades *et al*., 2013) como los marinados a base de vino tinto y salsa de soja podían prevenir el crecimiento de *S*. Enteritidis en ternera cruda almacenada durante 10 d a 5 ºC y 5 d a 15 ºC, y que el crecimiento de *Listeria* resultaba completamente inhibido, incluso en condiciones de abuso de temperatura (15 ºC).

En el presente trabajo (Tabla 29), la presurización redujo los niveles de viables totales en 2.14 unidades logarítmicas y la aplicación individual de las enterocinas A y B, pediocina PA-1 o nisina Z dio lugar a reducciones de 0.36, 0.55 y 0.28 unidades logarítmicas, respectivamente. La bacteriocina más efectiva fue la nisina comercial, que disminuyó los niveles de viables totales 1.18 unidades logarítmicas. El efecto de las altas presiones combinadas con la pediocina comercial produjo un efecto antimicrobiano sinérgico, con reducciones 0.88 unidades logarítmicas mayores que la suma de la inactivación obtenida con cada tratamiento individual. Durante el almacenamiento en refrigeración a 8 ºC, los niveles de viables totales aumentaron 0.94 unidades logarítmicas en las muestras de carpaccio no tratadas, probablemente debido al crecimiento de las BAL. Los géneros *Lactobacillus* y *Leuconostoc* son comunes en carpaccio de ternera envasado a vacío (Lucquin *et al*., 2012). En las muestras de carpaccio tratadas con nisina o pediocina comercial en combinación con las altas presiones, se observó un ligero efecto antimicrobiano sinérgico al final del período investigado. En el resto se detectó una recuperación de los viables totales. En un trabajo en que se combinaba la alta presión con bacteriocinas en pechuga de pavo y jamón curado (Pal *et al*., 2008), el aumento de la vida útil se atribuyó a un incremento de la fase de latencia bacteriana después de la aplicación de los tratamientos.

Tabla 26. Niveles (log ufc/g) de *L. monocytogenes* en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, bacteriocinas y sus combinaciones, mantenido durante 30 d a 8 ºC.

	0 h	1 d	3 d	7 d	15 d	30 d
Control	6.67 ± 0.30^{gB}	6.31 ± 0.51^{eA}	6.31 ± 0.74^{fgA}	6.49 ± 0.31^{hAB}	6.51 ± 0.31^{fAB}	6.40 ± 0.50^{fAB}
450 MPa	5.58 ± 0.53^{dE}	5.23 ± 0.97^{cD}	5.00 ± 0.83^{cdC}	5.07 ± 0.72^{cdCD}	4.11 ± 0.16^{A}	4.48 ± 1.20^{bB}
Enterocinas A y B	6.47 ± 0.41^{fgC}	5.77 ± 1.02^{dA}	6.08 ± 0.70^{fB}	6.20 ± 0.60^{fgB}	5.78 ± 0.68^{eA}	6.34 ± 0.47^{fBC}
Pediocina PA-1	6.03 ± 0.94^{eC}	5.93 ± 0.85^{dBC}	5.71 ± 1.04^{eA}	5.93 ± 0.54^{fBC}	5.77 ± 0.56^{eAB}	6.33 ± 0.40^{fD}
Nisina Z	6.57 ± 0.38^{fgB}	6.43 ± 0.45^{eAB}	6.37 ± 0.57^{gA}	6.42 ± 0.51^{ghAB}	6.37 ± 0.50^{fA}	6.47 ± 0.61^{fAB}
Nisina comercial	6.25 ± 0.50^{efE}	4.63 ± 0.23^{bA}	4.96 ± 0.36^{cdB}	5.40 ± 0.76^{eC}	4.59 ± 0.41^{dA}	5.66 ± 0.70^{dD}
Pediocina comercial	5.18 ± 0.94^{bcB}	4.58 ± 0.45^{bA}	4.91 ± 0.49^{cAB}	4.82 ± 0.35^{bcAB}	4.55 ± 0.26^{dA}	5.93 ± 0.28^{eC}
450 MPa + Ent. A y B	5.12 ± 0.62^{bcE}	4.60 ± 0.72^{bD}	4.06 ± 0.28^{bB}	4.74 ± 0.58^{bD}	3.69 ± 0.13^{bA}	4.25 ± 0.40^{bC}
450 MPa + Ped. PA-1	5.05 ± 0.99^{bE}	4.49 ± 0.54^{bC}	3.98 ± 0.02^{bA}	5.11 ± 0.37^{deE}	4.21 ± 0.42^{cB}	4.76 ± 0.81^{cD}
450 MPa + Nisina Z	5.49 ± 0.12^{cdD}	5.35 ± 0.17^{cD}	5.15 ± 0.13^{dC}	5.35 ± 0.15^{deD}	4.73 ± 0.42^{dB}	4.29 ± 0.11^{bA}
450 MPa + Nisina com.	4.90 ± 0.81^{bD}	4.55 ± 0.27^{bC}	4.00 ± 0.07^{bB}	4.69 ± 0.15^{bCD}	3.80 ± 0.41^{bAB}	3.59 ± 0.27^{aA}
450 MPa+ Ped. com.	4.19 ± 0.37^{aC}	3.83 ± 0.22^{aB}	3.70 ± 0.37^{aB}	3.70 ± 0.19^{aB}	3.23 ± 0.14^{aA}	3.65 ± 0.15^{aB}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 27. Niveles (log ufc/g) de *S.* Enteritidis en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, bacteriocinas y sus combinaciones, mantenido durante 30 d a 8 ºC.

	0 h	1 d	3 d	7 d	15 d	30 d
Control	5.76 ± 0.53^{eC}	5.55 ± 0.22^{fBC}	5.77 ± 0.50^{gC}	5.50 ± 0.51^{eBC}	4.86 ± 0.15^{efA}	5.35 ± 0.67^{efB}
450 MPa	1.63 ± 0.04^{aE}	1.56 ± 0.08^{cD}	1.61 ± 1.86^{bE}	0.45 ± 0.52^{aC}	0.35 ± 0.40^{bcB}	0.24 ± 0.28^{aA}
Enterocinas A y B	5.83 ± 0.28^{eC}	5.80 ± 0.05^{gC}	5.56 ± 0.28^{efB}	5.79 ± 0.08^{fC}	5.22 ± 0.23^{ghA}	5.81 ± 0.62^{hC}
Pediocina PA-1	5.93 ± 0.45^{efB}	5.99 ± 0.07^{gB}	5.61 ± 0.16^{fgA}	5.45 ± 0.14^{eA}	5.51 ± 0.30^{hiA}	5.58 ± 0.69^{gA}
Nisina Z	5.75 ± 0.30^{eBC}	5.93 ± 0.05^{gC}	5.58 ± 0.02^{efgBC}	5.59 ± 0.11^{efBC}	4.78 ± 0.44^{eA}	5.51 ± 1.31^{fgB}
Nisina comercial	5.83 ± 0.02^{eB}	5.77 ± 0.12^{fgB}	5.62 ± 0.36^{fgB}	5.12 ± 0.24^{dA}	5.15 ± 0.51^{fgA}	5.26 ± 1.63^{eA}
Pediocina comercial	6.16 ± 0.07^{fD}	5.95 ± 0.06^{gCD}	5.41 ± 0.59^{eAB}	5.41 ± 0.29^{eAB}	5.69 ± 0.12^{iBC}	5.30 ± 1.68^{eA}
450 MPa + Ent. A y B	3.24 ± 0.07^{dE}	2.10 ± 0.95^{dD}	2.11 ± 1.29^{cD}	1.80 ± 0.83^{cC}	1.05 ± 0.31^{dB}	0.64 ± 0.74^{bA}
450 MPa + Ped. PA-1	3.01 ± 0.28^{dE}	1.72 ± 0.38^{cD}	0.57 ± 0.31^{aB}	0.30 ± 0.35^{aA}	0.60 ± 0.35^{cBC}	0.66 ± 0.76^{bC}
450 MPa + Nisina Z	2.21 ± 0.98^{cF}	1.30 ± 0.25^{bC}	1.50 ± 0.01^{bD}	1.66 ± 0.95^{bcE}	0.30 ± 0.00^{abcA}	1.07 ± 1.23^{cdB}
450 MPa + Nisina com.	1.68 ± 1.60^{abD}	0.24 ± 0.28^{aB}	0.39 ± 0.45^{aB}	0.24 ± 0.28^{aB}	0.00 ± 0.00^{aA}	0.93 ± 1.08^{cC}
450 MPa + Ped. Com.	1.98 ± 1.94^{bcD}	3.28 ± 0.66^{eE}	3.90 ± 0.48^{dF}	1.51 ± 0.11^{bC}	0.15 ± 0.17^{abA}	1.14 ± 1.32^{dB}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 28. Niveles (log ufc/g) de *E. coli* O157:H7 en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, bacteriocinas y sus combinaciones, mantenido durante 30 d a 8 ºC.

	0 h	1 d	3 d	7 d	15 d	30 d
Control	6.23±0.48^{dA}	6.73±0.08^{efBC}	6.84±0.23^{dC}	6.62±0.11^{efBC}	6.51±0.29^{eAB}	6.24±0.40^{eA}
450 MPa	3.51±0.11^{abB}	4.26±0.79^{dC}	3.13±1.05^{bA}	4.06±0.26 ^{cC}	4.16±0.44^{dC}	3.69±0.14^{dB}
Enterocinas A y B	6.65±0.43^{eAB}	6.78±0.14^{efAB}	6.84±0.17^{dB}	6.70±0.18 ^{efAB}	6.63±0.25^{eAB}	6.58±0.26^{fA}
Pediocina PA-1	6.65±0.38^{eABC}	6.76±0.09^{efBC}	6.75±0.18^{dABC}	6.83±0.16^{fC}	6.56±0.32^{eAB}	6.52±0.41^{fA}
Nisina Z	6.70±0.15^{eBC}	6.57±0.16^{eBC}	6.65±0.08^{dBC}	6.16±0.21 ^{dA}	6.81±0.11^{eC}	6.52±0.15^{fB}
Nisina comercial	6.61±0.07^{eBC}	6.60±0.22^{eBC}	6.70±0.23^{dC}	6.46±0.29^{eA}	6.54±0.17^{eAB}	6.64±0.05^{fBC}
Pediocina comercial	6.62±0.10^{eA}	6.95±0.08^{fD}	6.80±0.07^{dBC}	6.68±0.12^{efAB}	6.69±0.04^{eAB}	6.84±0.10^{gCD}
450 MPa + Ent. A y B	3.35±0.14^{aB}	3.53±0.66^{bBC}	3.64±0.64^{cBC}	3.71±0.51^{bC}	3.60±1.28^{bBC}	1.73±0.26^{aA}
450 MPa + Ped. PA-1	3.74±0.66^{bC}	2.97±0.26^{aB}	3.32±0.46^{bB}	3.30±0.27^{aB}	3.06±0.24^{aB}	1.74±0.07^{aA}
450 MPa + Nisina Z	4.32±0.11^{cE}	4.10±0.40^{cdD}	3.70±0.09^{cB}	3.83±0.13^{bcBC}	3.97±0.21^{cdCD}	3.21±0.72^{cA}
450 MPa+ Nisina com.	3.64±1.73^{abD}	3.41±1.46^{bCD}	2.85±0.32^{aB}	3.34±0.21^{aC}	3.43±0.56^{bCD}	2.17±1.61^{bA}
450 MPa+ Ped. com.	4.42±0.97^{cE}	3.97±0.57^{cD}	3.88±0.39^{cCD}	3.57±0.29^{abB}	3.69±0.13^{bcBC}	3.12±0.99^{cA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 29. Niveles (log ufc/g) de viables totales en carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, bacteriocinas y sus combinaciones, mantenido durante 30 d a 8 ºC.

	0 h	1 d	7 d	15 d	30 d
Control	7.09 ± 0.45^{eA}	7.08 ± 0.24^{efA}	7.84 ± 0.35^{dB}	7.99 ± 0.64^{cB}	8.03 ± 0.58^{defB}
450 MPa	4.95 ± 1.04^{bA}	4.83 ± 1.05^{bA}	4.92 ± 1.08^{aA}	5.73 ± 1.68^{abA}	7.01 ± 1.09^{bcB}
Enterocinas A y B	6.73 ± 0.30^{eA}	6.69 ± 0.34^{deA}	8.28 ± 0.09^{dB}	8.57 ± 0.11^{cC}	8.59 ± 0.09^{fC}
Pediocina PA-1	6.54 ± 0.24^{deA}	6.81 ± 0.21^{deB}	8.10 ± 0.30^{dC}	8.39 ± 0.17^{cD}	8.44 ± 0.04^{efD}
Nisina Z	6.81 ± 0.47^{eB}	6.33 ± 0.17^{dA}	7.65 ± 0.49^{dC}	8.46 ± 0.08^{cD}	8.51 ± 0.07^{efD}
Nisina comercial	5.91 ± 0.33^{cdA}	6.54 ± 0.44^{deB}	6.46 ± 0.21^{cB}	6.51 ± 0.36^{bB}	7.40 ± 0.23^{bcdC}
Pediocina comercial	7.17 ± 0.63^{eA}	7.41 ± 0.48^{fA}	7.58 ± 0.12^{dA}	8.04 ± 0.48^{cB}	8.27 ± 0.21^{defB}
450 MPa + Ent. A y B	5.74 ± 0.17^{cB}	5.58 ± 0.17^{cAB}	5.43 ± 0.13^{abA}	6.43 ± 0.62^{bC}	8.14 ± 0.53^{defD}
450 MPa + Ped. PA-1	5.83 ± 0.22^{cdAB}	5.57 ± 0.19^{cA}	5.89 ± 0.04^{bcBC}	6.20 ± 0.35^{abC}	7.94 ± 0.55^{defD}
450 MPa + Nisina Z	5.57 ± 0.14^{bcA}	5.55 ± 0.19^{cA}	5.42 ± 0.21^{abA}	5.93 ± 0.48^{abB}	7.59 ± 0.60^{cdeC}
450 MPa + Nisina com.	4.17 ± 0.95^{aA}	3.94 ± 1.12^{aA}	5.53 ± 0.41^{abC}	5.11 ± 0.70^{aB}	6.04 ± 0.36^{aD}
450 MPa + Ped. com.	4.15 ± 1.13^{aA}	4.48 ± 1.38^{abB}	6.03 ± 0.12^{bcC}	6.00 ± 0.18^{abC}	6.61 ± 1.06^{abD}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

4.2.2. EFECTO DE LOS TRATAMIENTOS SOBRE LAS CARACTERÍSTICAS DEL CARPACCIO DE TERNERA

De los estudios realizados en carpaccio de ternera se desprende que los tratamientos de altas presiones aumentaron ligeramente los valores de pH del producto y la aplicación individual de pediocina PA-1, nisina comercial o la combinación de 450 MPa durante 5 min y nisina comercial produjo una disminución del mismo, inmediatamente después de la presurización. Después de 30 d a 8 ºC, el pH disminuyó en las muestras no tratadas, hecho atribuible a la actividad de las BAL, principalmente *Lactobacillus* y *Leuconostoc* (Lucquin *et al*., 2012). Los valores de a_w aumentaron en el carpaccio tratado con altas presiones, individualmente o en combinación con el SLP o con ALF. También se registró un aumento en los valores de a_w en el carpaccio tratado con enterocinas A y B, pediocina PA-1, nisina Z o con la combinación de 450 MPa durante 5 min y enterocinas A y B o nisina comercial. Sin embargo, las diferencias observadas no fueron suficientes para comprometer la seguridad microbiológica del producto (Tablas 30 a 33).

Tabla 30. Valores de pH y a_w del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5, 10 y 15 min, mantenido durante 30 d a 8 ºC.

		Control	450 MPa/5 min	450 MPa/10min	450 MPa/15min
pH	1 d	5.68 ± 0.08^{aB}	5.71 ± 0.07^{aA}	5.67 ± 0.04^{aA}	5.72 ± 0.01^{aA}
	15 d	4.87 ± 0.04^{aA}	5.74 ± 0.07^{bA}	5.70 ± 0.02^{bA}	5.80 ± 0.07^{bA}
	30 d	4.96 ± 0.15^{aA}	5.72 ± 0.38^{bA}	5.97 ± 0.13^{bB}	5.80 ± 0.12^{bA}
a_w	1 d	0.965 ± 0.002^{aA}	0.967 ± 0.000^{aA}	0.971 ± 0.002^{aA}	0.969 ± 0.003^{aA}
	15 d	0.966 ± 0.002^{aA}	0.972 ± 0.001^{bB}	0.976 ± 0.000^{cA}	0.973 ± 0.000^{bcA}
	30 d	0.965 ± 0.000^{aA}	0.968 ± 0.000^{abA}	0.972 ± 0.001^{bA}	0.973 ± 0.003^{bA}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 31. Valores de pH y a_w del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 30 d a 8 ºC.

		0 h	1 d	7 d	15 d	30 d
pH	Control	5.48 ± 0.13^{abD}	5.36 ± 0.05^{aBC}	5.41 ± 0.04^{dCD}	5.28 ± 0.06^{cAB}	5.22 ± 0.05^{bA}
	SLP	5.39 ± 0.09^{abC}	5.44 ± 0.05^{bD}	5.09 ± 0.02^{aA}	5.17 ± 0.17^{aB}	5.09 ± 0.01^{aA}
	ALF	5.50 ± 0.06^{bcC}	5.48 ± 0.12^{bcC}	5.22 ± 0.01^{bB}	5.23 ± 0.03^{bcB}	5.05 ± 0.04^{aA}
	450 MPa	5.59 ± 0.14^{cC}	5.31 ± 0.06^{aAB}	5.24 ± 0.02^{bA}	5.21 ± 0.02^{abA}	5.39 ± 0.10^{cB}
	450 MPa + SLP	5.38 ± 0.01^{aB}	5.52 ± 0.04^{cC}	5.08 ± 0.07^{aA}	5.34 ± 0.05^{dB}	5.35 ± 0.02^{cB}
	450 MPa + ALF	5.49 ± 0.02^{bcC}	5.58 ± 0.03^{dD}	5.34 ± 0.13^{cA}	5.42 ± 0.10^{eB}	5.49 ± 0.05^{dC}
a_w	Control	0.921 ± 0.001^{aBC}	0.922 ± 0.002^{aC}	0.918 ± 0.001^{aA}	0.919 ± 0.001^{aAB}	0.919 ± 0.001^{bA}
	SLP	0.924 ± 0.002^{abB}	0.923 ± 0.002^{aB}	0.921 ± 0.005^{bA}	0.924 ± 0.001^{bcB}	0.920 ± 0.000^{bcA}
	ALF	0.921 ± 0.001^{aC}	0.922 ± 0.001^{aC}	0.922 ± 0.001^{bC}	0.919 ± 0.003^{aB}	0.915 ± 0.002^{aA}
	450 MPa	0.926 ± 0.000^{bC}	0.926 ± 0.002^{bC}	0.922 ± 0.003^{bB}	0.922 ± 0.001^{bB}	0.919 ± 0.001^{bA}
	450 MPa + SLP	0.929 ± 0.001^{cC}	0.928 ± 0.001^{cC}	0.924 ± 0.001^{cB}	0.925 ± 0.002^{cB}	0.921 ± 0.001^{cA}
	450 MPa + ALF	0.934 ± 0.002^{dB}	0.933 ± 0.002^{dB}	0.934 ± 0.001^{dB}	0.933 ± 0.002^{dB}	0.924 ± 0.001^{dA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 32. Valores de pH del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, bacteriocinas y sus combinaciones, mantenido durante 30 d a 8 ºC.

	0 h	1 d	7 d	15 d	30 d
Control	5.44 ± 0.16^{cdC}	5.36 ± 0.17^{abBC}	5.16 ± 0.23^{abcAB}	5.05 ± 0.40^{abcA}	4.98 ± 0.25^{cdefA}
450 MPa	5.38 ± 0.10^{bcdB}	5.37 ± 0.12^{abB}	5.26 ± 0.21^{bcdB}	5.29 ± 0.38^{cB}	5.01 ± 0.18^{defA}
Enterocinas A y B	5.37 ± 0.11^{bcdC}	5.39 ± 0.11^{abC}	4.94 ± 0.23^{aB}	4.95 ± 0.53^{abB}	4.84 ± 0.08^{abcdA}
Pediocina PA-1	5.25 ± 0.06^{aC}	5.39 ± 0.20^{abD}	4.96 ± 0.10^{aB}	4.76 ± 0.29^{aA}	4.73 ± 0.23^{aA}
Nisina Z	5.38 ± 0.15^{bcdC}	5.40 ± 0.15^{abC}	5.04 ± 0.20^{abB}	4.94 ± 0.43^{abA}	4.86 ± 0.14^{abcdeA}
Nisina comercial	5.31 ± 0.17^{abC}	5.31 ± 0.14^{aC}	5.34 ± 0.08^{cdC}	5.15 ± 0.07^{bcB}	5.04 ± 0.16^{fA}
Pediocina comercial	5.37 ± 0.13^{bcdD}	5.32 ± 0.12^{aC}	5.29 ± 0.09^{cdC}	5.05 ± 0.05^{abcB}	4.82 ± 0.12^{abcA}
450 MPa + Ent. A y B	5.45 ± 0.14^{cdC}	5.43 ± 0.24^{abC}	5.29 ± 0.39^{cdB}	5.07 ± 0.25^{abcA}	5.04 ± 0.06^{fA}
450 MPa + Ped. PA-1	5.37 ± 0.10^{bcdCD}	5.39 ± 0.12^{abD}	5.30 ± 0.34^{cdC}	4.92 ± 0.38^{abB}	4.79 ± 0.14^{abA}
450 MPa + Nisina Z	5.47 ± 0.08^{dC}	5.46 ± 0.07^{bC}	5.35 ± 0.18^{cdB}	5.10 ± 0.10^{bcA}	5.02 ± 0.36^{efA}
450 MPa + Nis. com.	5.33 ± 0.16^{abB}	5.32 ± 0.17^{abB}	5.40 ± 0.10^{dB}	5.34 ± 0.12^{cB}	5.24 ± 0.37^{gA}
450 MPa + Ped. com.	5.36 ± 0.10^{bcBC}	5.40 ± 0.10^{abC}	5.39 ± 0.11^{dBC}	5.32 ± 0.13^{cB}	4.96 ± 0.32^{bcdefA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 33. Valores de a_w del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, bacteriocinas y sus combinaciones, mantenido durante 30 d a 8 ºC.

	0 h	1 d	7 d	15 d	30 d
Control	0.922 ± 0.006^{abBC}	0.926 ± 0.003^{abC}	0.920 ± 0.006^{bAB}	0.916 ± 0.007^{aA}	0.918 ± 0.004^{aA}
450 MPa	0.927 ± 0.004^{cdeA}	0.928 ± 0.004^{bA}	0.925 ± 0.004^{cdeA}	0.927 ± 0.005^{cdefA}	0.926 ± 0.005^{deA}
Enterocinas A y B	0.931 ± 0.004^{eD}	0.929 ± 0.003^{bcBC}	0.930 ± 0.002^{efCD}	0.928 ± 0.002^{defB}	0.924 ± 0.003^{cdA}
Pediocina PA-1	0.930 ± 0.003^{eD}	0.927 ± 0.007^{abBC}	0.928 ± 0.003^{cdefC}	0.925 ± 0.003^{bcdeB}	0.921 ± 0.004^{bcA}
Nisina Z	0.929 ± 0.003^{deB}	0.927 ± 0.001^{abB}	0.928 ± 0.005^{defB}	0.924 ± 0.002^{bcdA}	0.924 ± 0.003^{cdA}
Nisina comercial	0.922 ± 0.004^{abA}	0.928 ± 0.003^{bC}	0.924 ± 0.004^{bcdB}	0.922 ± 0.004^{bcA}	0.921 ± 0.005^{bcA}
Pediocina comercial	0.920 ± 0.005^{aD}	0.925 ± 0.009^{aE}	0.912 ± 0.013^{aA}	0.915 ± 0.010^{aB}	0.918 ± 0.004^{abC}
450 MPa + Ent. A y B	0.930 ± 0.003^{eB}	0.929 ± 0.004^{bcAB}	0.928 ± 0.003^{defA}	0.930 ± 0.003^{efAB}	0.928 ± 0.003^{eAB}
450 MPa + Ped. PA-1	0.924 ± 0.005^{abcA}	0.928 ± 0.003^{bB}	0.931 ± 0.004^{fC}	0.931 ± 0.004^{fC}	0.928 ± 0.001^{eB}
450 MPa + Nisina Z	0.925 ± 0.002^{bcdA}	0.928 ± 0.005^{abB}	0.930 ± 0.003^{fC}	0.932 ± 0.003^{fC}	0.926 ± 0.001^{deA}
450 MPa + Nis. com.	0.931 ± 0.002^{eB}	0.931 ± 0.005^{cB}	0.929 ± 0.005^{efA}	0.931 ± 0.003^{fB}	0.932 ± 0.001^{fB}
450 MPa + Ped. com.	0.923 ± 0.004^{abcB}	0.927 ± 0.004^{abC}	0.923 ± 0.005^{bcB}	0.921 ± 0.009^{bA}	0.923 ± 0.003^{cAB}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Los tratamientos de altas presiones a 450 MPa durante 5, 10 o 15 min aumentaron la luminosidad (*L**) del carpaccio (Tabla 34). Este efecto se observó durante el período de refrigeración investigado, con una tendencia creciente en las muestras presurizadas durante 15 min. El aumento del valor de *L** en carne de ternera ya ha sido descrito (Carlez *et al*., 1995; Jung *et al*., 2003; Marcos *et al*., 2010) cuando los tratamientos de presión se aplicaron a temperaturas superiores a 0 ºC atribuyéndose, como se ha indicado anteriormente, a la desnaturalización de la globina y/o a la liberación o desplazamiento del grupo hemo (Carlez *et al*., 1995). Las altas presiones podrían aumentar el índice de luz reflejada respecto a la luz absorbida de la superficie de la carne, y según Goutefongea *et al*. (1995), este aumento podría estar relacionado con la coagulación de las proteínas sarcoplásmicas y miofibrilares.

Los valores de la tendencia al rojo (*a**) disminuyeron en el carpaccio tratado por alta presión ($P<0.05$) tras la aplicación de los tratamientos. Los valores más bajos se detectaron al final del período de refrigeración en las muestras presurizadas a 450 MPa durante 15 min. La disminución de los valores de *a** en los productos cárnicos presurizados se ha relacionado con algunos cambios en la molécula de mioglobina, tales como la oxidación de la mioglobina ferrosa a mioglobina férrica a presiones superiores a 400 MPa (Carlez *et al*., 1995). Franceschini *et al*. (2005) observaron una disminución de los valores de *a** en carpaccio de ternera tratado a 600 MPa durante 10 min. Sin embargo, esta disminución es menos severa en los productos cárnicos crudos curados debido a la adición de nitratos/nitritos, de manera que las carnes crudas curadas han presentado problemas de decoloración, con un aumento de *L** pero con menores efectos sobre *a** debido a la acción protectora del óxido nítrico (procedente del nitrito o nitrato sódico) sobre la mioglobina. Se cree que la nitrosilmioglobina ferrosa es un importante antioxidante en las carnes curadas (Andersen *et al*., 1990).

Los valores de la tendencia al amarillo (*b**) disminuyeron ($P<0.05$) en el carpaccio presurizado en comparación con las muestras control, aunque estas diferencias se atenuaron durante el almacenamiento a 8 ºC. Realini *et al*. (2011) observaron que la congelación (-35 ºC) del carpaccio de cerdo sometido a alta presión protegía el color de este producto cárnico, evitándose el aumento de *L** y la disminución de *a**, aunque la *b** sí resultó afectada por la presurización. Según estos autores, el mecanismo responsable de este efecto protector podría ser explicado en términos de desnaturalización reversible de la mioglobina inducida por la presión. En la carne fresca de ternera presurizada, la desnaturalización de la mioglobina sería intensa e irreversible. En cambio, este efecto sería más leve y reversible en la carne de ternera

congelada a temperaturas inferiores a 0 ºC. Además, la mioglobina recuperaría su conformación nativa y consecuentemente, el color normal de la carne fresca (Fernández *et al*., 2007). También Szerman *et al*. (2011) comprobaron que la congelación del carpaccio de ternera reducía los efectos perjudiciales de las altas presiones sobre los parámetros del color. Según estos autores, este hecho podría atribuirse a la minimización de la desnaturalización de las proteínas sarcoplásmicas y miofibrilares.

Tabla 34. Parámetros de color del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5, 10 y 15 min, mantenido durante 30 d a 8 ºC.

		Control	450 MPa/5 min	450 MPa/10 min	450 MPa/15 min
*L**	1 d	23.65±3.62^{aA}	31.26±2.82^{bA}	33.87±3.00^{bA}	33.71±2.07^{bA}
	15 d	25.29±1.64^{aA}	33.52±2.19^{bB}	35.95±3.17^{cA}	36.21±2.41^{cB}
	30 d	25.58±1.66^{aA}	31.17±2.06^{bA}	35.08±2.53^{cA}	36.19±1.76^{cB}
*a**	1 d	33.47±4.66^{cC}	18.05±1.79^{bB}	14.43±2.63^{aAB}	15.59±1.65^{abB}
	15 d	21.00±2.88^{cB}	18.40±1.95^{bB}	14.90±2.26^{aB}	14.77±2.03^{aB}
	30 d	17.58±2.12^{dA}	14.63±1.16^{cA}	12.43±2.01^{bA}	10.23±1.77^{aA}
*b**	1 d	26.53±3.97^{bB}	15.03±1.96^{aB}	13.50±1.36^{aA}	12.95±0.97^{aB}
	15 d	14.01±1.78^{bA}	14.28±1.21^{bB}	13.07±1.24^{abA}	12.31±0.96^{aAB}
	30 d	11.90±1.31^{aA}	12.55±1.00^{aA}	12.34±1.40^{aA}	11.49±1.07^{aA}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

En el carpaccio tratado a 450 MPa durante 5 min en combinación con el SLP o ALF se observó también un aumento ($P<0.05$) del valor de *L**, inmediatamente después de la presurización (Tabla 35). Este efecto de empalidecimiento originado por las altas presiones ha sido explicado por cambios moleculares, como se ha indicado anteriormente, aunque la modificación del color de los productos curados podía considerarse como aceptable (De Alba *et al*., 2012a, b; 2013a). Según Ferrini *et al*. (2012) estos cambios dependen del contenido de agua y de la a_w de los mismos.

Tabla 35. Parámetros de color del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 30 d a 8 ºC.

		0 h	7 d	15 d	30 d
*L**	Control	34.32 ± 2.90^{bA}	32.24 ± 3.82^{aA}	31.85 ± 1.32^{aA}	33.33 ± 3.50^{aA}
	SLP	34.79 ± 1.71^{bA}	35.63 ± 3.07^{bcA}	34.18 ± 3.87^{abA}	33.49 ± 2.47^{aA}
	ALF	30.92 ± 1.24^{aA}	34.12 ± 2.05^{abBC}	32.99 ± 2.91^{abAB}	35.80 ± 2.59^{aC}
	450 MPa	37.31 ± 1.71^{cA}	37.03 ± 1.75^{bcA}	38.34 ± 3.05^{cA}	36.22 ± 2.22^{aA}
	450 MPa + SLP	38.22 ± 2.47^{cA}	37.56 ± 1.91^{cA}	35.86 ± 1.60^{bcA}	36.06 ± 2.83^{aA}
	450 MPa + ALF	38.00 ± 2.72^{cB}	35.54 ± 1.56^{bcA}	35.47 ± 0.97^{bcA}	35.40 ± 1.44^{aA}
*a**	Control	19.58 ± 1.98^{abAB}	20.39 ± 3.90^{bB}	18.36 ± 2.25^{bcAB}	17.34 ± 2.93^{abA}
	SLP	17.73 ± 3.22^{abA}	17.97 ± 3.15^{abA}	19.38 ± 3.53^{cA}	16.37 ± 4.96^{aA}
	ALF	20.32 ± 2.03^{bB}	17.47 ± 2.53^{abA}	19.80 ± 3.36^{cAB}	17.41 ± 2.69^{abA}
	450 MPa	17.61 ± 1.96^{abAB}	18.01 ± 1.61^{abB}	15.78 ± 2.58^{abA}	20.29 ± 1.84^{bC}
	450 MPa + SLP	17.11 ± 2.78^{aA}	16.81 ± 2.65^{aA}	14.70 ± 2.06^{aA}	16.22 ± 2.79^{aA}
	450 MPa + ALF	17.69 ± 2.61^{abAB}	16.58 ± 2.76^{aA}	18.06 ± 1.52^{bcAB}	19.55 ± 3.22^{abB}
*b**	Control	7.19 ± 1.08^{aB}	9.16 ± 3.68^{bC}	6.33 ± 1.24^{aAB}	4.70 ± 0.97^{aA}
	SLP	6.82 ± 1.35^{aAB}	7.52 ± 2.07^{abB}	7.16 ± 2.31^{aAB}	5.10 ± 2.37^{aA}
	ALF	6.73 ± 1.31^{aB}	6.62 ± 1.53^{aB}	7.09 ± 1.63^{aB}	4.90 ± 1.35^{aA}
	450 MPa	7.61 ± 0.82^{aAB}	8.08 ± 1.43^{abB}	6.04 ± 1.69^{aA}	8.30 ± 2.07^{cB}
	450 MPa + SLP	7.18 ± 1.27^{aB}	7.23 ± 1.42^{abB}	6.48 ± 2.02^{aAB}	5.45 ± 1.17^{abA}
	450 MPa + ALF	7.44 ± 1.38^{aA}	7.43 ± 1.54^{abA}	7.35 ± 1.26^{aA}	7.28 ± 2.38^{bcA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

En el presente trabajo se observó una disminución del valor de *L** en las muestras tratadas con ALF inmediatamente después de la presurización, pero no se observó efecto del SLP sobre la luminosidad. Al final del período de refrigeración a 8 ºC, los valores de *L** no cambiaron significativamente, excepto en las muestras tratadas con ALF aplicada individualmente o en combinación con las altas presiones, registrándose un aumento y una disminución en los valores de este parámetro, respectivamente. Inmediatamente después de la presurización, la aplicación individual del SLP no afectó a la tendencia al rojo (*a**) ni al amarillo (*b**) del carpaccio de ternera. Después de 30 d a 8 ºC se observó un aumento ($P<0.05$) de *a** en las muestras presurizadas y una disminución ($P<0.05$) en las muestras tratadas con ALF. Respecto a la tendencia al amarillo, se observó una disminución de *b** ($P<0.05$) en las muestras no tratadas, en las tratadas con ALF y en aquellas tratadas con el SLP en combinación con las altas presiones al final del período investigado. Al igual que

pudimos observar en el jamón curado loncheado (Tabla 13), la generación *in situ* de H_2O_2 por la GOX y la glucosa añadidas no afectó a los parámetros del color del carpaccio de ternera.

La adición de bacteriocinas al carpaccio de ternera no afectó a los valores de *L** (Tabla 36). Sin embargo, se observó un aumento en los valores de este parámetro con todos los tratamientos combinados de bacteriocinas y altas presiones a 450 MPa durante 5 min, aunque fue significativamente ($P<0.05$) mayor en las muestras presurizadas en combinación con la pediocina PA-1 o con la nisina comercial, respectivamente. Después de 30 d a 8 ºC, los valores de *L** disminuyeron ($P<0.05$) en las muestras tratadas con enterocinas A y B y aumentaron ($P<0.05$) en las muestras tratadas con pediocina comercial.

Inmediatamente después de la presurización, la tendencia al rojo (*a**) disminuyó ($P<0.05$) con la presurización aplicada individualmente o en combinación con las bacteriocinas. Solo con nisina Z se observó la disminución ($P<0.05$) de los valores de *a**. Sin embargo, las diferencias en *a** tendían a atenuarse con todos los tratamientos al final del almacenamiento.

En cuanto a la tendencia al amarillo (*b**) se observó una disminución de los valores en las muestras presurizadas o en aquellas combinadas con las bacteriocinas, aunque esta disminución fue significativa ($P<0.05$) solo con pediocina PA-1 o pediocina comercial combinadas con altas presiones. Después de 30 d a 8 ºC, las diferencias en los valores de *b** tendían a atenuarse. Liu *et al*. (2012) observaron que la combinación de altas presiones a 200 o 400 MPa durante 10 min y enterocina LM-2 a 256 o 2560 UA/g tuvieron poca influencia sobre los valores de *L** y *b** en jamón cocido loncheado almacenado durante 90 d a 4 ºC. Sin embargo, un día después de la presurización, todos los tratamientos combinados con la excepción de enterocina LM-2 a 256 UA/g en combinación con 200 MPa, redujeron los valores de *a** comparados con las muestras no tratadas. Además, los valores de *a** tendían a disminuir en todas las muestras tratadas con el tiempo de almacenamiento. De los resultados obtenidos en el presente trabajo se desprende que los cambios detectados en el carpaccio de ternera fueron principalmente debidos al efecto de las altas presiones y al tiempo de almacenamiento, sin que las bacteriocinas tuvieran una influencia reseñable en las características del producto. Debido al hecho de que el color de la superficie del alimento es el primer parámetro de calidad evaluado por los consumidores, y que es crítico para la aceptabilidad del producto (Leon *et al*., 2006), las tecnologías de higienización empleadas en alimentos deberían afectar mínimamente a esta característica.

Tabla 36. Parámetros de color del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, bacteriocinas y sus combinaciones, mantenido durante 30 d a 8 ºC.

		0 h	7 d	15 d	30 d
*L**	Control	32.82±2.97^{abcdA}	33.64±2.67^{abcdA}	33.67±2.65^{abcdA}	32.59±2.50^{bcA}
	APH	35.08±3.66^{defAB}	35.84±3.01^{eB}	35.55±2.42^{dB}	33.75±2.19^{bcdA}
	Ent. A y B	32.38±3.47^{abB}	33.18±2.34^{aB}	32.68±1.78^{abB}	30.17±2.50^{aA}
	Ped. PA-1	32.62±3.03^{abcA}	33.26±1.34^{abA}	31.82±1.95^{aA}	31.75±2.84^{abA}
	Nisina Z	32.00±2.79^{aA}	33.88±3.45^{abcdB}	31.90±2.11^{aA}	31.67±2.10^{abA}
	Nisina com.	33.30±2.63^{abcdeA}	34.44±0.98^{abcdeB}	32.38±1.74^{aA}	33.46±2.22^{bcdAB}
	Ped. Com.	32.89±2.63^{abcdA}	33.48±2.68^{abcA}	33.39±3.26^{abcA}	35.47±3.83^{deB}
	APH+ Ent AB	34.48±3.39^{bcdefA}	35.37±2.38^{cdeA}	35.07±2.12^{cdA}	35.09±2.93^{deA}
	APH + PA-1	35.20±2.82^{efA}	35.48±2.44^{deA}	35.46±2.51^{dA}	34.29±2.64^{cdeA}
	APH + Nis. Z	34.79±2.94^{cdefAB}	35.12±2.58^{bcdeB}	34.50±1.93^{bcdAB}	33.62±1.71^{bcdA}
	APH + Nis.	35.83±1.96^{fA}	35.96±2.00^{eA}	35.30±1.47^{dA}	35.98±2.35^{eA}
	APH + Ped.	34.89±2.00^{cdefA}	36.30±1.58^{eB}	34.62±2.24^{cdA}	35.38±2.03^{deAB}
*a**	Control	22.33±2.82^{fB}	21.13±3.77^{defB}	19.23±3.66^{aA}	22.66±3.79^{deB}
	APH	17.82±2.66^{abA}	17.27±3.88^{abA}	20.20±3.65^{aB}	19.65±3.70^{abcB}
	Ent. A y B	23.48±2.8^{fB}	19.40±6.06^{bcdA}	20.29±3.11^{aA}	23.07±4.69^{eB}
	Ped. PA-1	22.93±3.20^{fB}	21.99±3.84^{efAB}	20.28±1.96^{aA}	20.38±2.02^{abcdA}
	Nisina Z	20.01±1.95 ^{cdA}	20.04±6.15^{cdeA}	20.22±3.87^{aA}	20.12±4.44^{abcA}
	Nisina com.	22.22±3.07^{fA}	22.65±2.31^{fA}	21.26±2.99^{aA}	21.70±3.21^{bcdeA}
	Ped. com.	21.94±3.07^{efA}	20.79±4.62^{defA}	23.86±2.34^{bB}	21.89±4.78^{cdeA}
	APH + Ent AB	18.31±2.59^{abcA}	19.21±2.65^{bcdAB}	20.71±2.84^{aB}	18.94±3.69^{aAB}
	APH + PA-1	16.67±1.76^{aA}	16.64±3.70^{aA}	19.84±2.43^{aB}	20.26±2.72^{abcB}
	APH + Nis. Z	18.62±2.99^{bcdAB}	17.76±2.56^{abcA}	19.97±2.96^{aB}	19.66±2.32^{abcB}
	APH + Nis.	20.30±2.52^{deB}	18.70±2.17^{abcdA}	20.91±1.97^{aB}	20.46±3.02^{abcdB}
	APH + Ped.	19.75±2.18^{cdA}	18.76±2.00^{abcdA}	18.82±3.18^{aA}	19.57±3.13^{abA}
*b**	Control	12.78±5.31^{bcdB}	9.80±2.45^{cdeA}	8.16±2.96^{aA}	12.06± 5.78^{efB}
	APH	10.48±3.46^{abB}	8.52±2.06^{abcA}	10.01±2.95^{abcAB}	9.81±4.08^{abcdAB}
	Ent. A y B	13.95±3.98^{dB}	9.75±2.53^{cdeA}	8.51±3.16^{abA}	13.09±6.98^{fB}
	Ped. PA-1	13.47±4.23^{cdC}	10.62±1.30^{eB}	8.39±2.64^{abA}	11.07±5.18^{bcdefB}
	Nisina Z	10.33±2.31^{abBC}	10.09±3.97^{deB}	8.52±4.20^{abA}	11.59±6.58^{cdefC}
	Nisina com.	12.72±6.77^{bcdC}	10.14±4.13^{deB}	8.58±2.42^{abA}	9.04±2.63^{abAB}
	Ped. com.	12.28±6.46^{abcdB}	9.45±4.36^{bcdeA}	11.24±2.50^{cAB}	10.78±4.74^{abcdeAB}
	APH + Ent AB	10.63±3.49^{abAB}	9.29±1.40^{abcdeA}	10.20±3.71^{bcAB}	11.11±5.56^{bcdefB}
	APH + PA-1	9.92±1.75^{aB}	8.53±1.39^{abcA}	9.24±3.09^{abcAB}	11.69±5.03^{defC}
	APH + Nis. Z	11.12±4.00^{abcB}	8.89±1.26^{abcdA}	8.93±3.21^{abA}	11.50±4.86^{cdefB}
	APH + Nis.	11.05±5.32^{abcC}	7.93±2.39^{aA}	9.12±2.42^{abAB}	9.46±2.39^{abcB}
	APH + Ped.	10.14±4.55^{aB}	8.24±2.79^{abA}	8.48±1.82^{abA}	8.60±2.02^{aA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

En relación a la textura (Tabla 37), la fuerza al corte (N) disminuyó (P<0.05) en el carpaccio presurizado a 450 durante 5, 10 y 15 min, inmediatamente después del tratamiento, aunque las diferencias fueron menores a mayor tiempo de presurización. Por otro lado, no se detectaron diferencias significativas en la fuerza máxima después de los tratamientos entre el carpaccio tratado por altas presiones y el no presurizado. Durante la conservación a 8 ºC ambos parámetros incrementaron su valor en el carpaccio presurizado.

Tabla 37. Propiedades de textura del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5, 10 y 15 min.

		Control	450 MPa/5 min	450 MPa/10 min	450 MPa/15 min
Fuerza al corte (N)	1 d	46.19±14.11^{cA}	21.03±5.61^{aA}	29.09±4.11^{abA}	35.51±9.35^{bcA}
	15 d	32.33±10.10^{aA}	35.87±8.46^{aB}	44.59±21.61^{aB}	43.00±6.53^{aA}
	30 d	40.76±18.67^{aA}	37.15±12.69^{aB}	66.29±8.55^{bC}	65.76±11.61^{bB}
Fuerza máxima (N)	1 d	66.71±32.02^{aA}	62.02±10.45^{aA}	71.56±23.32^{aA}	75.72±21.49^{aA}
	15 d	91.50±29.75^{aA}	97.83±49.18^{aA}	77.10±23.63^{aA}	85.32±28.48^{aA}
	30 d	79.11±16.92^{aA}	154.77±36.97^{cB}	134.12±38.62^{bcB}	96.59±41.34^{abA}

Los valores son la media ± SD. Las medias dentro de la misma fila con diferentes superíndices en minúscula difieren significativamente a P<0.05. Las medias dentro de la misma columna con diferentes superíndices en mayúscula difieren significativamente a P<0.05.

La alta presión podría afectar a la integridad de los lisosomas y aumentar la actividad de la catepsina D y de la fosfatasa ácida en carne de ternera presurizada, influyendo sobre sus características texturales (Jung *et al*., 2000 a, b). De acuerdo con Ma y Ledward (2004), los cambios en las proteínas contráctiles miofibrilares son responsables de las diferencias de las propiedades de textura de la carne tratada por alta presión.

En el presente trabajo, la fuerza al corte no se vio afectada por los tratamientos de alta presión a 450 MPa, el SLP, ALF, o sus combinaciones, excepto por el tratamiento de presurización combinado con el SLP que originó un aumento (P<0.05) de este parámetro, inmediatamente después de la presurización (Tabla 38). Después de 30 d a 8 ºC, los valores de la fuerza al corte aumentaron (P<0.05) con la aplicación individual de los antimicrobianos y disminuyeron (P<0.05) con el tratamiento combinado entre altas presiones y el SLP, en comparación con el tiempo 0 h.

Szerman *et al*. (2011) no observaron diferencias significativas en los valores de la fuerza al corte de las muestras de carpaccio congeladas y tratadas a 400, 500 o 600 MPa durante 5 min a 0 y 5 ºC o descongeladas y presurizadas con los mismos tratamientos a 5 y 20 ºC, respecto a sus respectivos controles. Según Vaudagna *et al*. (2012), los tratamientos de presurización de 400 y 650 MPa durante 1 y 5 min a -30 ºC, no afectaron significativamente a los valores de la fuerza al corte del carpaccio congelado o de acuerdo con nuestros resultados, inmediatamente después de la presurización, la dureza del carpaccio de ternera determinada como fuerza máxima no se vio afectada por los tratamientos (Tabla 38). Después de 30 d a 8 ºC, se observó un aumento (P<0.05) de esta propiedad en las muestras tratadas con el SLP individualmente.

En el carpaccio sometido a altas presiones en combinación con bacteriocinas no se observaron diferencias significativas en la fuerza al corte inmediatamente después de la presurización (Tabla 39). Después de 30 d a 8 ºC, este parámetro aumentó (P<0.05) en las muestras tratadas con enterocinas A y B, pediocina PA-1, nisina Z, pediocina comercial, y en las muestras presurizadas combinadas con nisina Z o nisina comercial. Respecto a la dureza, solo aumentó significativamente (P<0.05) en las muestras tratadas con pediocina comercial individualmente o en combinación con la alta presión, inmediatamente después de la presurización. Al final del período investigado, este parámetro aumentó (P<0.05) en las muestras no tratadas y en las tratadas con enterocinas A y B individualmente o combinadas con altas presiones. En el jamón curado tratado con altas presiones, nisina, pediocina comercial o sus combinaciones no se observó una tendencia clara de ambas propiedades de textura durante la refrigeración del producto. La dureza del jamón cocido loncheado disminuyó por las altas presiones a 400 MPa durante 10 min en combinación con enterocina LM-2, respecto a las muestras tratadas a 200 MPa y enterocina o a las muestras control tras la aplicación de los tratamientos (Liu *et al*., 2012). Según estos autores, la dureza, jugosidad y la aceptabilidad general del jamón cocido tratado y sin tratar disminuyeron durante el almacenamiento en refrigeración a 4 ºC.

De acuerdo con los trabajos publicados, las altas presiones causan desnaturalización proteica, agregación o gelificación, que puede dar lugar a la tenderización o endurecimiento de la carne, en función del sistema proteico cárnico, la temperatura, la presión y su duración (Sun y Holley, 2010).

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Tabla 38. Propiedades de textura del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, el SLP, ALF y sus combinaciones, mantenido durante 30 d a 8 ºC.

		0 h	7 d	15 d	30 d
Fuerza al corte (N)	Control	23.22±6.65^{aA}	26.01±7.27^{aA}	22.85±2.41^{aA}	25.54±9.01^{aA}
	SLP	28.37±6.35^{aA}	34.84±3.66^{aB}	25.26±2.95^{abA}	40.43±8.18^{bB}
	ALF	23.84±8.80^{aA}	30.23±7.83^{aAB}	25.56±6.25^{abAB}	32.69±7.06^{abB}
	450 MPa	30.36±7.87^{aA}	26.15±8.55^{aA}	33.37±5.77^{cA}	29.81±6.39^{aA}
	450 MPa + SLP	43.92±14.74^{bB}	29.99±9.79^{aA}	28.80±4.45^{bcA}	30.32±7.76^{aA}
	450 MPa + ALF	30.28±4.94^{aA}	35.47±11.46^{aA}	31.83±6.76^{cA}	32.18±8.08^{abA}
Dureza (N)	Control	121.11±31.47^{aAB}	91.31±14.33^{aA}	100.97±10.98^{aA}	137.03±56.82^{abB}
	SLP	107.95±24.89^{aA}	115.80±17.24^{abA}	128.59±33.53^{abAB}	163.81±13.03^{bB}
	ALF	91.96±14.24^{aA}	119.10±23.62^{abA}	119.78±27.00^{abA}	106.00±23.68^{aA}
	450 MPa	111.52±41.58^{aAB}	154.83±35.44^{cB}	127.03±14.81^{abAB}	103.32±34.51^{aA}
	450 MPa + SLP	117.88±19.07^{aA}	132.72±15.59^{bcA}	114.91±14.49^{abA}	131.62±38.16^{abA}
	450 MPa + ALF	103.60±30.99^{aA}	130.52±15.56^{bcAB}	154.39±22.68^{bB}	108.36±33.66^{aA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

Tabla 39. Propiedades de textura del carpaccio de ternera tratado con altas presiones a 450 MPa durante 5 min, bacteriocinas y sus combinaciones, mantenido durante 30 d a 8 ºC.

		0 h	7 d	15 d	30 d
Dureza (N)	Control	107.6±34.0^{abA}	114.0±45.5^{abcAB}	131.6 ± 43.9 ^{bcdAB}	139.9±48.7^{abcB}
	450 MPa	132.5±46.4^{bcA}	140.9±60.4^{cdA}	129.8±31.6^{bcdA}	149.6±37.8^{bcA}
	Enterocinas A y B	82.0.±31.5^{aA}	121.7±46.8^{abcdB}	120.7±25.7^{abcdB}	119.2±29.3^{abB}
	Pediocina PA-1	78.5±30.7^{aA}	83.9±24.5^{aA}	93.6±22.3^{abA}	103.3±33.3^{aA}
	Nisina Z	132.6±36.1^{bcBC}	156.5±47.2^{dC}	106.8±27.3^{abA}	127.4±34.4^{abAB}
	Nisina com.	138.7±41.8^{bcA}	130.9±33.4^{bcdA}	149.4±47.3^{cdA}	154.3±31.1^{bcA}
	Pediocina com.	154.9±55.5^{cB}	91.2±18.3^{abA}	156.5±72.7^{dB}	179.3±62.8^{cB}
	450 MPa+ Ent. A y B	104.1±47.7^{abA}	143.8±37.1^{cdB}	82.9±24.5^{aA}	133.9±45.2^{abB}
	450 MPa + Ped. PA-1	109.7±25.3^{abA}	139.7±48.2^{cdA}	131.7±43.8^{bcdA}	117.4±39.6^{abA}
	450 MPa + Nisina Z	100.8±28.2^{abA}	152.81±35.22^{cdB}	93.8±16.4^{abA}	105.6±30.2^{aA}
	450 MPa + Nis. com.	118.9±43.4^{abcA}	123.9±36.3^{abcdA}	126.1±36.4^{bcdA}	142.4±45.2^{abcA}
	450 MPa + Ped. com.	158.0±32.1^{cB}	114.1±16.9^{abcA}	109.8±30.1^{abcA}	150.0±33.4^{bcB}
Fuerza al corte (N)	Control	33.4±16.1^{abA}	31.7±9.3^{abA}	29.2±7.3^{aA}	34.9±9.8^{aA}
	450 MPa	29.1±9.9^{aA}	34.1±13.0^{bBC}	38.7±8.6^{bcdC}	32.1±7.8^{aAB}
	Enterocinas A y B	28.4±8.6^{aA}	35.9±14.2^{bB}	38.7±10.2^{bcdB}	36.7±9.2^{aB}
	Pediocina PA-1	33.2±8.9^{abAB}	28.8±11.7^{abA}	39.2±14.7^{bcdB}	50.0±18.8^{bC}
	Nisina Z	30.0±7.3^{aA}	30.6±10.7^{abA}	35.2±6.9^{abcdAB}	39.3±12.9^{aB}
	Nisina com.	29.9±12.9^{aA}	30.4±10.9^{abA}	39.9±9.5^{cdB}	33.6±11.5^{aAB}
	Pediocina com.	28.2±9.1^{aA}	33.7±14.2^{bAB}	32.6±10.6^{abAB}	36.5±7.8^{aB}
	450 MPa + Ent. A y B	34.9±12.4^{abA}	31.9±6.7^{abA}	34.8±10.4^{abcdA}	36.7±6.7^{aA}
	450 MPa + Ped. PA-1	41.0±10.7^{bB}	33.2±8.3^{bA}	32.3±9.7^{abA}	39.2±12.5^{aAB}
	450 MPa + Nisina Z	27.6±8.3^{aA}	25.5±7.0^{aA}	41.2±16.6^{dB}	46.7±15.8^{bC}
	450 MPa + Nis. com.	31.1±6.0^{aA}	31.8±7.1^{abAB}	36.3±13.0^{abcdBC}	37.8±8.8^{aC}
	450 MPa + Ped. com.	33.8±8.1^{abA}	34.6±8.2^{bA}	33.0±7.5^{abcA}	32.8±8.9 ^{aA}

Los valores son la media ± SD. Las medias dentro de la misma columna con diferentes superíndices en minúscula difieren significativamente a $P<0.05$. Las medias dentro de la misma fila con diferentes superíndices en mayúscula difieren significativamente a $P<0.05$.

4.3. TRATAMIENTOS COMBINADOS DE ALTAS PRESIONES Y NITRITO EN LA INACTIVACIÓN DE PATÓGENOS

Por último, se ha investigado el efecto de nuevos tratamientos combinados de nitrito sódico y altas presiones en la inactivación de *E. coli* y *L. monocytogenes* en sistemas tampón considerando la posibilidad de su posterior aplicación en productos cárnicos. Se emplearon concentraciones de 0.25, 0.50, 0.75, 1.0, 2.0 y 3.0 mM de nitrito sódico acidificado a pH 4.0 (Figuras 1 y 2). Las células de ambos patógenos fueron sensibles al nitrito sódico. La exposición de *E. coli* a 1.0, 2.0 y 3.0 mM de nitrito sódico acidificado causó una reducción inmediata en los recuentos de viables inferior a una unidad logarítmica pero después del almacenamiento a 4 ºC durante 24 h, las reducciones aumentaron 1.10, 2.60 y 5 unidades logarítmicas, respectivamente. La presencia de 0.25, 0.50 y 0.75 mM de nitrito sódico acidificado causó reducciones de *L. monocytogenes* de 0.90, 2.30 y 3.60 unidades logarítmicas, respectivamente, después de 24 h a 4 ºC (De Alba *et al*., 2013b).

La aplicación del tratamiento de 300 MPa durante 8 min causó una reducción de 1.40 unidades logarítmicas de *E. coli*. Cuando la presurización se combinó con 1.0 ó 2.0 mM de nitrito sódico, el número de viables disminuyó 2.40 y 4.90 unidades logarítmicas, respectivamente, mientras que con 3.0 mM de nitrito sódico, los niveles de *E. coli* se situaron por debajo del límite de detección (<2.4 unidades logarítmicas), indicando reducciones de más de 6 unidades logarítmicas. El grado de inactivación, 24 h más tarde, fue superior a 6 unidades logarítmicas con todos los tratamientos combinados (Figura 1). El tratamiento de presión de 225 MPa durante 8 min causó una reducción de una unidad logarítmica en la viabilidad de *L. monocytogenes*. Con los tratamientos de alta presión en combinación con 0.25, 0.50 y 0.75 mM de nitrito sódico, se obtuvieron reducciones de 1.50, 2.70 y 4.20 unidades logarítmicas. Veinticuatro horas más tarde de la aplicación de los tratamientos se observó un aumento (P<0.001) de la inactivación, alcanzando reducciones de 3.40, 6.10 y 6.10 unidades logarítmicas, respectivamente (Figura 2).

El efecto antimicrobiano de la combinación de nitrito sódico y las altas presiones resultó sinérgico sobre *E. coli* y *L. monocytogenes*. Con *E. coli* se alcanzaron más de 4 reducciones logarítmicas en la primera hora de exposición, resultando a continuación el grado de inactivación menos acusado. Esta disminución del efecto sinérgico frente a *E. coli* durante el almacenamiento podría atribuirse a la disminución del número de viables entre las células tratadas por presión, las tratadas con nitrito sódico acidificado y las no presurizadas que permanecían en el tampón ácido. La pérdida de viabilidad de las células de *E. coli* y de *L. monocytogenes*

dañadas por presión mantenidas en tampón ácido o zumo de frutas ha sido estudiada por García-Graells *et al*. (1998) y Jordan *et al*. (2001). Jofré *et al*. (2010) observaron que las células de *S. aureus* dañadas por el tratamiento de alta presión morían posteriormente cuando se inoculaban en un medio que contenía sal o nitrito. Sin embargo, no se han publicado trabajos sobre el efecto bactericida sinérgico de la presión combinada con nitrito acidificado.

Figura 1. Inactivación de *L. monocytogenes* a pH 4.0 mediante altas presiones (225 MPa durante 8 min), nitrito y sus combinaciones.

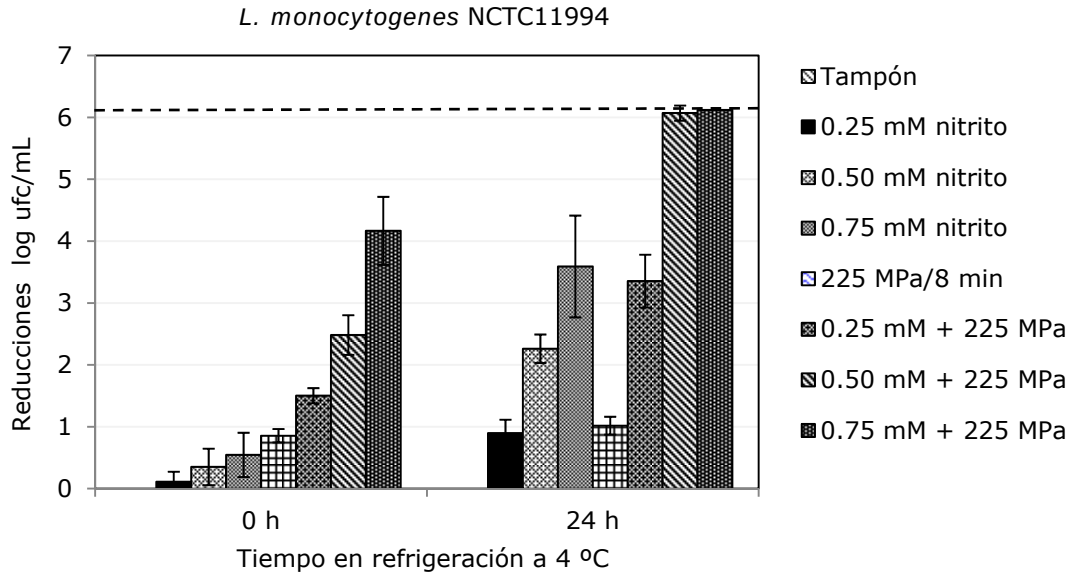
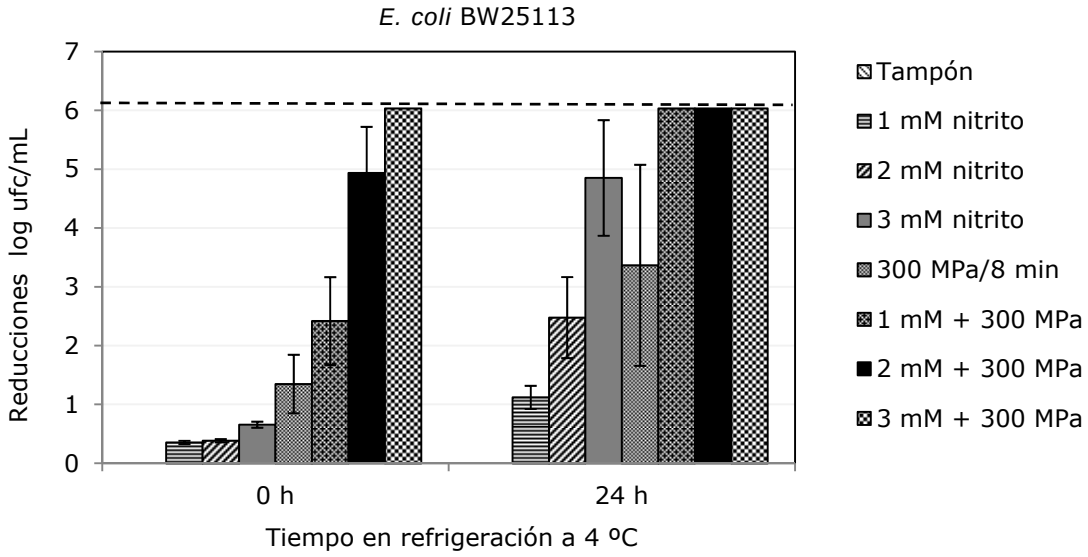
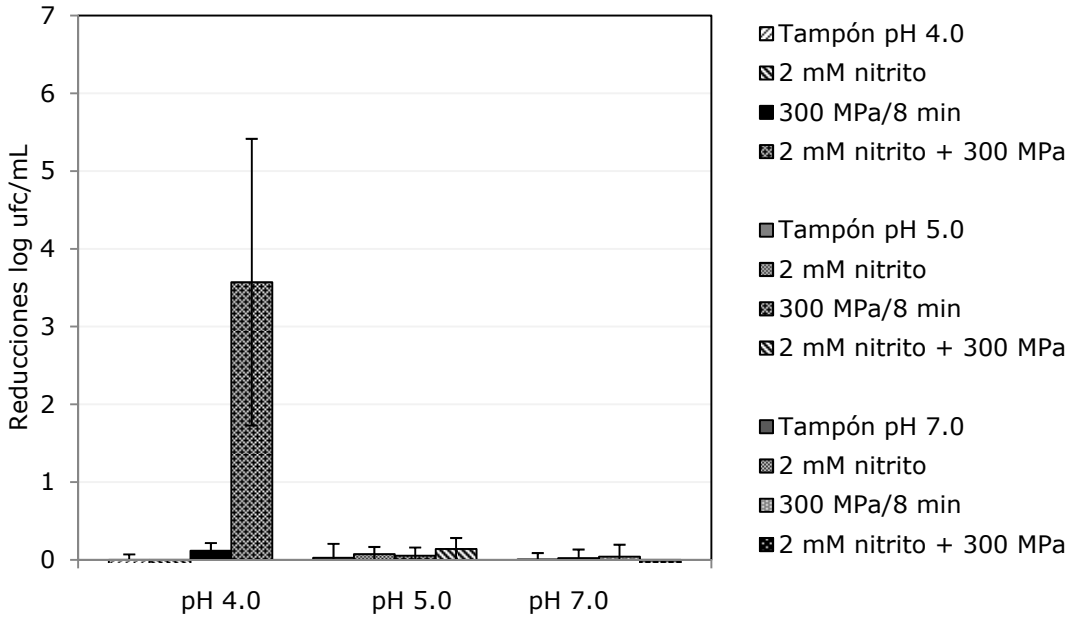


Figura 2. Inactivación de *E. coli* a pH 4.0 mediante altas presiones (300 MPa durante 8 min), nitrito y sus combinaciones.



A continuación se estudió el efecto del pH sobre la inactivación de *E. coli* por altas presiones combinadas con 2 mM de nitrito sódico acidificado a pH 4.0 (Figura 3), detectándose reducciones de aproximadamente 4 unidades logarítmicas inmediatamente después de la presurización, pero no se observaron efectos antimicrobianos a pH 5.0 ni a pH 7.0. No obstante, a pH 4.5 se observó un efecto antimicrobiano sinérgico ligero con la combinación de las altas presiones y 3 mM de nitrito sódico. Bajo estas condiciones, el tratamiento combinado causó una reducción de 1.40 unidades logarítmicas comparado con las 0.23 unidades logarítmicas obtenidas con la suma de los tratamientos por separado. Se demuestra así, la dependencia del pH con el efecto bactericida del nitrito.

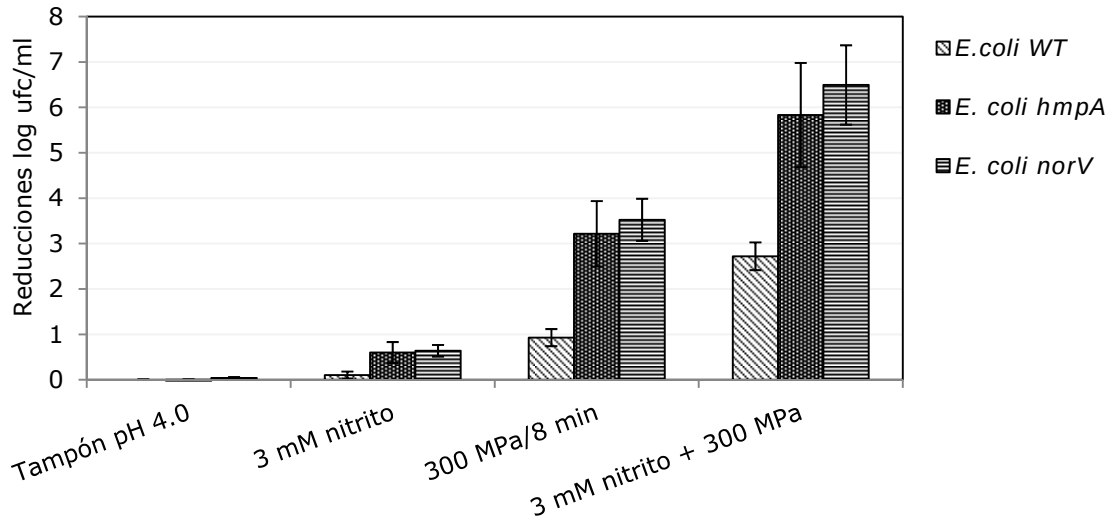
Figura 3. Efecto del pH sobre la inactivación de *E. coli* mediante altas presiones (300 MPa durante 8 min) en combinación con nitrito.



Con objeto de profundizar en el efecto combinado, se investigó con los mutantes *hmp*A y *nor*V, carentes de las enzimas protectoras dioxigenasa del óxido nítrico y de la reductasa del óxido nítrico, respectivamente (Gardner *et al*., 2002; Poole, 2005) (Figura 4) de *E. coli* BW25113. Estos mutantes fueron significativamente más sensibles al nitrito acidificado que la cepa parental, e incluso más sensibles al tratamiento de alta presión aplicado individualmente. Además, las diferencias en los efectos antimicrobianos sinérgicos detectadas entre las cepas no fueron estadísticamente significativas. El mecanismo del efecto sinérgico podría ser debido a la inactivación de los sistemas protectores frente al estrés nitrosativo o al aumento de la formación de productos reactivos del oxígeno (ROS) como el peroxinitrito

durante el tratamiento de presión. Sin embargo, Domitrovic *et al*. (2003) indicaron un efecto diferente del nitrito sódico sobre la resistencia a la presión, encontrando que niveles bajos de óxido nítrico liberados desde los compuestos donadores protegían a *Saccharomyces cerevisiae* frente a las altas presiones debido a una respuesta frente al estrés provocada por el óxido nítrico. El fundamento del aumento de la sensibilidad a la presión de los mutantes *hmp*A y *nor*V no se conoce, incluso en ausencia de nitrito acidificado. Una posible explicación podría ser que la presión pudiera causar disrupción de los procesos metabólicos celulares, originando un aumento de la producción interna del óxido nítrico.

Figura 4. Efecto de las mutaciones que afectan al estrés nitrosativo sobre la inactivación de *E. coli* por nitrito acidificado a pH 4.0, aplicado individualmente o en combinación con altas presiones (300 MPa durante 8 min).



De acuerdo con los resultados obtenidos, sería útil ensayar posibles efectos antimicrobianos sinérgicos de tratamientos combinados entre altas presiones y nitrito en alimentos como carnes fermentadas o algunos quesos, en los que el nitrito es un aditivo permitido, con el objetivo de disminuir la cantidad de producto añadido. Los nitratos y nitritos inhiben el desarrollo microbiano indeseable, sobre todo del patógeno *C. botulinum*, principal motivo por el que las autoridades de ciertos países autorizan su empleo. Los nitritos se añaden a los productos cárnicos como tales o en forma de nitratos. Participan en la formación del color, del sabor y del aroma y tienen efectos antioxidantes y antimicrobianos. A pesar de los efectos beneficiosos existen una serie de problemas asociados a su uso como son la formación de nitrosaminas, las cuales poseen potencial carcinogénico, por lo que su uso está limitado. No obstante, estos problemas alcanzan mayor importancia en productos cárnicos en los

que se aplica un tratamiento térmico elevado y de forma especial, los sometidos a fritura para su consumo. La cantidad de nitritos disminuye con el tiempo o llega a desaparecer en productos cárnicos de larga maduración, pero en principio no desaparecen las sustancias cancerígenas. Cuanta más cantidad de nitratos y nitritos se empleen mayores podrán ser los peligros asociados para la salud de los consumidores. Por tanto, se impone la necesidad de utilizarlos en cantidades mínimas para conseguir los efectos beneficiosos y reducir al máximo los perjudiciales. La combinación con altas presiones podría ser una alternativa de interés.

5. Conclusiones

5. CONCLUSIONES

Los tratamientos de alta presión a 500 y 600 MPa durante 5 min resultaron eficaces para inactivar *S*. Enteritidis en jamón curado loncheado. No se registró recuperación del patógeno durante el almacenamiento posterior en condiciones de abuso de temperatura.

La combinación de 400 o 500 MPa durante 10 min con nisina comercial mostró un efecto antimicrobiano sinérgico sobre *E. coli* O157:H7 en jamón curado loncheado. Este efecto se mantuvo con el tratamiento combinado de 500 MPa y nisina durante el almacenamiento del producto.

La combinación de 450 MPa durante 10 min con el sistema lactoperoxidasa o lactoferrina produjo un efecto antimicrobiano sinérgico frente a *S*. Enteritidis en jamón curado loncheado. El efecto alcanzado con la combinación de 450 MPa y el sistema lactoperoxidasa fue sinérgico al final de la refrigeración frente a *L. monocytogenes*.

Los cambios en la textura y color del jamón curado fueron poco acusados. La presurización produjo un ligero aumento de la opacidad y una menor tendencia al rojo, así como alguna modificación de la textura. Dichos cambios tendieron a atenuarse durante el almacenamiento.

Los tratamientos de 450 MPa durante 5, 10 y 15 min inactivaron *S*. Enteritidis en carpaccio de ternera refrigerado. La reducción de *Salmonella* alcanzó 5 unidades logarítmicas con el tratamiento menos intenso.

El tratamiento de 450 MPa durante 5 min combinado con el sistema lactoperoxidasa o lactoferrina activada resultaron eficaces en la inactivación de *S*. Enteritidis y *E. coli* O157:H7 en carpaccio de ternera. La combinación de 450 MPa y el sistema lactoperoxidasa mostró un efecto antimicrobiano sinérgico frente a *S*. Enteritidis y *E. coli* O157:H7.

La presurización del carpaccio de ternera a 450 MPa durante 5 min o su combinación con las enterocinas A y B, pediocina PA-1, nisina Z, nisina o pediocina comercial inactivaron *S*. Enteritidis y *E. coli* O157:H7. En el caso de *E. coli* O157:H7, el efecto antimicrobiano de la alta presión combinada con las enterocinas A y B, pediocina PA-1 o nisina comercial fue sinérgico.

Los tratamientos modificaron ligeramente el color y la textura del carpaccio de ternera con un ligero aumento de la opacidad y una menor tendencia al rojo. La

dureza aumentó al final del período de refrigeración con los tratamientos más intensos.

Se comprobó el efecto bactericida del nitrito aplicado individualmente o en combinación con las altas presiones sobre *E. coli* y *L. monocytogenes* en estudios *in vitro.* El efecto antimicrobiano combinado fue sinérgico a pH 4.0 frente a ambos microorganismos.

Los mutantes *hmp*A y *nor*V de *E. coli* BW25113 fueron más sensibles al nitrito acidificado y al tratamiento de alta presión que la cepa parental apuntando a la participación del óxido nítrico en el efecto bactericida.

6. Conclusions

CONCLUSIONS

High hydrostatic pressure at 500 or 600 MPa for 5 min were effective on the inactivation of *S*. Enteritidis in sliced dry-cured ham. A recovery of the pathogen was not detected during the storage of the product in temperature abuse conditions.

The combined treatment of 400 or 500 MPa for 10 min and commercial nisin showed a synergistic antimicrobial effect against *E. coli* O157:H7 in sliced dry-cured ham. This effect was evident kept during the storage of the product when 500 MPa and commercial nisin were combined.

The combination of 450 MPa for 10 min and the lactoperoxidase system or lactoferrin caused a synergistic antimicrobial effect against *S*. Enteritidis in sliced dry-cured ham. The antimicrobial effect achieved with the combination of 450 MPa and the lactoperoxidase system was synergistic at the end of the refrigerated period against *L. monocytogenes*.

Changes on texture and color of dry-cured ham were slight. Pressurization caused a slight increase of lightness and lower redness, as well as some modifications of texture. Such changes tended to attenuate during storage.

Treatments at 450 MPa for 5, 10 and 15 min were effective on the inactivation of *S*. Enteritidis in refrigerated beef carpaccio. A reduction of 5 log units in *Salmonella* counts was achieved with the least intense treatment.

The combined treatments at 450 MPa for 5 min and the lactoperoxidase system or activated lactoferrin were effective on the inactivation of *S*. Enteritidis and *E. coli* O157:H7 in beef carpaccio. The combination of 450 MPa and the lactoperoxidase system showed a synergistic antimicrobial effect against *S*. Enteritidis and *E. coli* O157:H7.

Pressurization of beef carpaccio at 450 MPa for 5 min or the combination of this high pressure treatment with enterocins A and B, pediocin PA-1, nisin Z, commercial nisin or pediocin were effective to inactivate *S*. Enteritidis and *E. coli* O157:H7. In case of *E. coli* O157:H7, a synergistic antimicrobial effect was detected when the high pressure treatment and enterocins A and B, pediocin PA-1 or commercial nisin were combined.

Treatments modified slightly color and texture of beef carpaccio, with a slight increase of lightness and lower redness. Hardness increased at the end of the refrigerated period with the most intense treatments.

A bactericidal effect of sodium nitrite applied alone or in combination with high pressure was observed against *E. coli* and *L. monocytogenes in vitro* studies. The combined antimicrobial effect was synergistic at pH 4.0 against both microorganisms.

The *E. coli* BW25113 *hmp*A and *nor*V mutants were more sensitive to acidified nitrite and high pressure than *E. coli* BW25113 wild type pointing at the involvement of nitric oxide in the bactericidal effect.

7. Bibliografía

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8. Resumen ampliado [1]

[1]Este resumen ampliado se presenta en cumplimiento de las directrices de la normativa de desarrollo del Real Decreto 99/2011, de 28 de enero, que regula los estudios de doctorado en la Universidad Complutense de Madrid (UCM) (BOUC nº 14, de 21 de diciembre de 2012) y de acuerdo con las especificaciones establecidas por la Comisión de Doctorado de la UCM.

INTRODUCCIÓN

El estilo de vida de la sociedad actual en los países desarrollados ha originado una serie de cambios en los hábitos alimentarios de los consumidores. La tendencia hacia alimentos menos tratados pero más estables y seguros, con una vida útil más larga, más fáciles de preparar, nutritivos y elaborados sin la adición de conservantes químicos, puede conducir a una pérdida de la conservación intrínseca de los alimentos y de la protección a través del procesado.

Atendiendo a las demandas de los consumidores, la industria alimentaria ha transformado alimentos ya procesados en productos listos para el consumo (o RTE, del inglés Ready-To-Eat). Según el Reglamento 2073/2005, los alimentos listos para el consumo se definen como aquellos alimentos destinados al consumo directo sin necesidad de cocinado u otro tipo de transformación eficaz para eliminar o reducir a un nivel aceptable los microorganismos peligrosos (CE, 2005). En la mayoría de los casos, los productos RTE son envasados a vacío o en atmósferas modificadas/protectoras y han de mantenerse en refrigeración hasta su consumo con el fin de alargar su vida útil.

Las formas de procesado de los alimentos RTE que implican una reducción de tamaño y conllevan la manipulación del alimento en operaciones como el loncheado, troceado, dosificación y envasado, incrementan los riesgos de una contaminación accidental, que puede verse acentuada si el almacenamiento de estos productos se realiza en condiciones de abuso o aumento incontrolado de temperatura (Cava *et al.*, 2009; Garrido *et al.*, 2010).

Los alimentos RTE pueden contaminarse durante su preparación con microorganismos patógenos, procedentes del ambiente, utillaje empleado en las operaciones, manipuladores, etc. Por este motivo, debe mantenerse en todo momento la cadena de frío y deben manipularse siguiendo las normas higiénicas correspondientes. Los microorganismos patógenos más frecuentes son diversos serovares de *Escherichia coli* (incluido el O157:H7) y de *Salmonella* spp., *Campylobacter jejuni*, *Listeria monocytogenes*, *Yersinia enterocolitica* y *Staphylococcus aureus*. Entre estos patógenos, *L. monocytogenes* y *Salmonella* spp. pueden considerarse ubicuos por lo que se detectan con cierta frecuencia en una gran variedad de alimentos. *L. monocytogenes* es el patógeno que más preocupa entre los que se multiplican incluso en condiciones de refrigeración estricta, sin sobrepasar 4 ºC. *E. coli* O157:H7 posee una dosis infectiva muy baja y es responsable de un proceso patológico muy severo. *S. aureus* es un microorganismo

oportunista, cuyo crecimiento en un alimento mantenido en refrigeración suele quedar condicionado a un abuso de la temperatura de almacenamiento.

La implantación de buenas prácticas de fabricación y la aplicación de los principios del sistema de análisis de peligros y puntos de control críticos (APPCC) deben garantizar la seguridad de los alimentos. Sin embargo, pueden no resultar suficientes. La aplicación de tecnologías no térmicas como las altas presiones hidrostáticas (APH), los pulsos de luz, las radiaciones, etc., y determinados agentes biológicos (solos o en combinación con alguna de las tecnologías anteriormente mencionadas) como los ácidos orgánicos, las bacteriocinas y otros agentes antimicrobianos, se presentan como una herramienta eficaz para conseguir la higienización de los alimentos RTE.

Según los criterios de seguridad alimentaria para los distintos patógenos, el patrón universal para *Salmonella* spp. y *E. coli* O157:H7 es de "tolerancia cero" (ausencia en 25 g). Sin embargo, el criterio para *L. monocytogenes* en alimentos RTE difiere entre países. Para el Departamento de Agricultura de EE.UU. (USDA) es de la categoría "tolerancia cero" pero para la Unión Europea (CE, 2007) varía dependiendo de la población receptora y del tipo de alimento. Para la población normal, el criterio es de 100 ufc/g siempre que las listerias no se multipliquen durante el almacenamiento. El reglamento de la UE considera que pertenecen a esta categoría, los productos RTE de pH≤4.4, los de a_w≤0.92, los de pH≤5.0 y a_w≤0.94 y los de vida útil inferior a 5 días.

En la UE y por lo tanto en España, los criterios microbiológicos vigentes se rigen por el Reglamento (CE) 2073/2005 (CE, 2005) así como por sus sucesivas modificaciones: el Reglamento (CE) 1441/2007 (CE, 2007), el Reglamento (UE) 365/2010 (UE, 2010) y el Reglamento (UE) 1086/2011 (UE, 2011).

Según el último informe de la Autoridad Europea de Seguridad Alimentaria (EFSA) publicado en 2013 (EFSA, 2013), la incidencia de *Salmonella* y *L. monocytogenes* ha disminuido durante los últimos años, a diferencia de *E. coli* verocitotoxigénico (VTEC) que ha aumentado desde 2008.

La combinación de técnicas tradicionales (temperatura, a_w, potencial redox, etc.) y otras estrategias de conservación (envasado en atmósferas modificadas, bacteriocinas, ácidos orgánicos, cultivos bioprotectores, APH, etc.), podrían establecer una serie de barreras más efectivas contra las bacterias alterantes y patógenas. En un determinado alimento se pueden utilizar múltiples barreras u obstáculos (Leistner, 2000) para controlar el crecimiento microbiano, pudiendo actuar de forma aditiva o sinérgica. Aun cuando cada alimento o proceso nuevo o

modificado originará nuevas condiciones para los microorganismos, utilizando el concepto de barreras u obstáculos, se deberán obtener alimentos más seguros para los consumidores.

En el presente trabajo, se propone la aplicación de las altas presiones hidrostáticas (APH), al ser una tecnología de conservación no térmica encaminada a aumentar la seguridad microbiológica y la vida útil de los alimentos, en combinación con distintas estrategias de bioconservación (Stiles, 1996) como las bacteriocinas, el sistema lactoperoxidasa (SLP) y la lactoferrina (LF), en un intento de potenciar posibles sinergias y minimizar la intensidad de los tratamientos aplicados sobre dos productos cárnicos RTE, jamón curado loncheado y carpaccio de ternera. Se han evaluado además, los cambios producidos como consecuencia de los tratamientos en las características físicoquímicas, reológicas y el color de estos productos. Esta tecnología cuenta con un buen grado de aceptación por parte del consumidor, lo que ha llevado a una mayor implantación a nivel industrial con respecto a otras tecnologías emergentes.

Los niveles de intensidad de aplicación del tratamiento por la industria cárnica oscilan entre 400-600 MPa con tiempos cortos de presurización de entre 3 y 7 minutos a temperatura ambiente. Estas condiciones de tratamiento conducen, en la mayoría de los casos, a un nivel de inactivación de aproximadamente 4 unidades logarítmicas para la mayoría de los microorganismos patógenos y alterantes (Bajovic *et al.*, 2012).

En general, los cambios inducidos en el color por las altas presiones varían en función de diferentes parámetros, entre ellos, el contenido de mioglobina, siendo estos cambios más intensos en carne roja fresca que en carne blanca y productos cárnicos curados. El efecto negativo sobre el color puede limitarse optimizando variables como la intensidad y el tiempo de presurización, la temperatura, el curado, la disponibilidad de oxígeno y el pH. La influencia de las altas presiones sobre la textura depende del rigor-mortis de la carne, de los niveles de presión, de la temperatura (Ma *et al.*, 2004) y de la duración del tratamiento (Sun y Holley, 2010).

Las bacteriocinas son polipéptidos o proteínas cuyo tamaño oscila entre 900 y 5800 Daltons, sintetizadas en los ribosomas, que en su forma madura ejercen un efecto antimicrobiano contra bacterias estrechamente relacionadas con las bacterias productoras. Las bacteriocinas son producidas por bacterias Gram-negativas y Gram-positivas. Pueden proporcionar a sus productores, inmunes a los productos antibacterianos que sintetizan, una ventaja adaptativa frente a otras bacterias. Generalmente, eliminan la fuerza protónica que atraviesa las membranas

bacterianas, al formar pequeños poros en éstas, pero también pueden inhibir la síntesis de proteínas, la formación de ARN-m y la duplicación del ADN. Aunque las células de las bacterias Gram-negativas son resistentes a las bacteriocinas de las bacterias lácticas, el estrés químico o físico puede deteriorar la estructura lipopolisacarídica de la membrana externa y hacerlas sensibles a la acción de las bacteriocinas.

En microbiología de alimentos, las bacteriocinas producidas por las bacterias lácticas son de especial interés porque normalmente dirigen su efecto bactericida a diferentes bacterias Gram-positivas alterantes y a bacterias patógenas, y bajo condiciones de estrés químico (ácidos orgánicos, agentes quelantes, etc.) o físico (pH, congelación, APH, etc.) pueden dirigir su efecto bactericida contra diferentes Gram-negativas importantes para los alimentos (Fang y Tsai, 2003; Kalchayanand *et al.*, 1992, 1998; Stevens *et al.*, 1991). Sin embargo, hay que tener en cuenta que la producción de una bacteriocina en medios sintéticos de laboratorio no implica su efectividad en un sistema alimentario. La carne y los productos cárnicos son sistemas complejos en los que puede producirse una pérdida parcial o total de la actividad bacteriocina, pudiendo ésta verse afectada por el procesamiento o las condiciones de almacenamiento del alimento, por cambios en la solubilidad y en la carga, por unirse a componentes del alimento o por ser destruidas por proteasas (Aasen *et al.*, 2003).

La nisina es una bacteriocina producida por algunas cepas de *Lactococcus lactis* y *Streptococcus* sp. Se han descrito diferentes tipos de nisina, entre ellas, la nisina A, Z, Q y F producidas por *L. lactis* y la nisina U y U2 producidas por *S. uberis* 42 y *S. agalactiae* D536, que difieren entre sí en algunos residuos de aminoácidos. Es la única bacteriocina reconocida como GRAS (Generally Regarded As Safe) por la FDA (Food and Drug Administration, EE.UU.), permitiéndose su uso comercial (O´Keeffe y Farrell, 2000) en más de 50 países, incluyendo EE.UU. y algunos países de la Unión Europea. La nisina se encuentra dentro de la lista de aditivos aprobados con el número E234 (95/2/EC), sin efectos adversos conocidos. Pertenece a la clase Ia dentro de la clasificación de Klaenhammer (1993) y evita la germinación de las esporas de Gram-positivos de los géneros *Clostridium* y *Bacillus* (Savadogo *et al.*, 2006), mostrando también eficacia frente a organismos Gram-positivos de los géneros *Staphylococcus*, *Listeria*, *Enterococcus*, etc.

La pediocina PA-1 es producida por *Pediococcus acidilactici*, aunque puede ser producida por otras especies del mismo género e incluso por diferentes géneros de bacterias lácticas (Miller *et al.*, 2005). Pertenece a la clase IIa de las bacteriocinas (Klaenhammer, 1993) y es activa frente a algunas especies de los géneros

Lactococcus, *Lactobacillus*, *Carnobacterium*, *Enterococcus*, *Bacillus*, *Brochothrix*, *Clostridium*, *Listeria* y *Staphylococcus*, aunque la sensibilidad depende de la especie e incluso de la cepa. Presenta actividad inhibitoria frente a *L. monocytogenes* (Nielsen *et al.*, 1990).

Las enterocinas son bacteriocinas producidas por bacterias pertenecientes al género *Enterococcus*. Entre las enterocinas descritas se encuentran las enterocinas A y B, AS-48, I, L50A y L50B, CCM 4231, 13, 416K1, etc. (Khan *et al.*, 2010). Las enterocinas A y B son producidas por algunas cepas de *Enterococcus faecium* y pertenecen a la clase IIa de las bacteriocinas (Klaenhammer, 1993). La enterocina A fue aislada de la cepa *E. faecium* CTC 492 y la enterocina B de la cepa *E. faecium* T136. Ambas actúan sinérgicamente y son activas frente a un amplio espectro de bacterias Gram-positivas que incluyen especies de *Clostridium*, *Propionibacterium*, *Listeria*, *Staphylococcus* y la mayoría de bacterias lácticas. Diferentes estudios han demostrado que las enterocinas A y B son efectivas frente a *L. monocytogenes*, *Salmonella* y *S. aureus* inoculados en productos cárnicos (Garriga *et al.*, 2002) y proporcionan protección extra cuando se rompe la cadena de frío (Marcos *et al.*, 2008).

El sistema lactoperoxidasa (SLP) forma parte de los mecanismos de defensa de la inmunidad innata en los mamíferos. Está presente en diferentes fluidos biológicos y está constituido por tres componentes: la enzima lactoperoxidasa (LPX), el ión tiocianato (SCN^-) y el peróxido de hidrógeno (H_2O_2), siendo estos dos últimos necesarios para su activación. La LPX cataliza la oxidación del SCN^- por el H_2O_2, generando compuestos intermediarios activos con propiedades antimicrobianas, que ejercen su acción sobre grupos sulfhidrilos de enzimas y otras proteínas presentes en las membranas bacterianas. Para activar el SLP es necesaria la presencia de H_2O_2 que puede ser generado enzimáticamente mediante la glucosa oxidasa (GOX). La presencia de GOX junto con su sustrato permite que el H_2O_2 requerido por el SLP sea generado de forma continua (Seifu *et al.*, 2005). El sistema SLP-GOX estaría constituido por las enzimas LPX y GOX, glucosa y una fuente de iones isotiocianato. Su acción está influida por diversos factores, como la especie bacteriana, el pH del medio, el tiempo de incubación, la temperatura y la densidad celular (Björck *et al.*, 1975).

La lactoferrina (LF) es una glicoproteína de aproximadamente 80 kDa de peso molecular que pertenece a la familia de las transferrinas. El efecto bacteriostático de la LF parece estar relacionado con su capacidad de secuestrar hierro, mientras que su actividad bactericida parece debida a su unión con los componentes de la pared

celular o alteración de los mismos, tales como moléculas de lipopolisacáridos en bacterias Gram-negativas y ácidos teicoicos o lipoteicoicos en Gram-positivas. Como consecuencia, se produce despolarización, pérdida de la integridad de la membrana y del gradiente de pH (Vorland *et al.,* 1999). La LF posee derivados tales como su forma amidada (AMILF) y sus formas purificada (lactoferricina, LFC) y no purificada (PDLF), digeridas con pepsina. Debido a sus propiedades antibacterianas de amplio espectro, han sido propuestas como conservantes naturales en los alimentos (Naidu, 2002).

OBJETIVOS

Los objetivos de la presente tesis doctoral se centran en conseguir la máxima seguridad microbiológica de productos cárnicos listos para el consumo (RTE) mediante tratamientos combinados de altas presiones y bioconservación.

En este contexto, se plantearon los siguientes objetivos parciales:

1.- Inactivar a los patógenos *L. monocytogenes*, *S. enterica* ser Enteritidis y *E. coli* O157:H7 en jamón curado loncheado y en carpaccio de ternera mediante tratamientos de alta presión aplicados individualmente o en combinación con bacteriocinas, el sistema lactoperoxidasa o lactoferrina.

2.- Evaluar los cambios en las características fisicoquímicas, reológicas y en el color de los productos como consecuencia de los tratamientos de inactivación.

3.- Investigar *in vitro* nuevos tratamientos combinados de nitrito sódico y altas presiones para la inactivación de *L. monocytogenes* y *E. coli*.

RESULTADOS

Los tratamientos de 400, 500 y 600 MPa durante 5 min fueron efectivos en la inactivación de *S. enterica* ser. Enteritidis inoculada en jamón curado loncheado mantenido durante 60 d a 8 ºC, alcanzándose mayores reducciones logarítmicas con los tratamientos de presión más intensos. Las células dañadas por la presurización no fueron capaces de recuperarse durante el almacenamiento, incluso bajo condiciones de abuso de temperatura. *Salmonella* no se detectó por recuento directo en el jamón tratado a 600 MPa a partir de los 7 d de almacenamiento, aunque sí se determinó su presencia en 20 g de producto tras realizar el enriquecimiento de las muestras. Los niveles de *Salmonella* disminuyeron gradualmente en las muestras de jamón no presurizado a lo largo del período de refrigeración investigado, siendo incapaz de crecer a 8 ºC debido a la baja a_w y alto contenido de NaCl existentes en el jamón curado.

La oxidación lipídica aumentó con la presurización y el almacenamiento y el contenido en aminoácidos libres fue similar en las muestras control y en las tratadas por las altas presiones después de 60 d de almacenamiento a 8 ºC. Respecto al color del jamón curado presurizado, la luminosidad (L^*) aumentó en el jamón tratado con 500 y 600 MPa durante 5 min en comparación con las muestras no tratadas y las presurizadas a 400 MPa. El valor de L^* disminuyó ($P<0.001$) durante el almacenamiento en el jamón control y en el tratado, y los cambios observados durante el período de refrigeración fueron mayores que los inducidos por la presurización. Los valores de la tendencia al rojo (a^*) fueron menores en el jamón tratado a 500 y 600 MPa. Esta diferencia se mantuvo durante 60 d de almacenamiento a 8 ºC y los valores más bajos se obtuvieron al final del período en refrigeración. Los valores de la tendencia al amarillo (b^*) resultaron también significativamente afectados ($P<0.001$) por los tratamientos de alta presión. Después de 24 h, las muestras tratadas a 600 MPa mostraron valores significativamente menores que el resto, presurizadas y no presurizadas. La tendencia al amarillo disminuyó ($P<0.05$) durante el almacenamiento, registrándose los valores más bajos en el jamón presurizado a 400 MPa y 600 MPa después de 60 d a 8 ºC.

En relación a la textura del jamón curado, los tratamientos de presurización redujeron la resistencia al corte en las muestras de jamón curado presurizado a 500 o 600 MPa durante 5 min comparadas con las muestras no presurizadas y las sometidas a los tratamientos menos intensos a 400 MPa. Respecto a la fuerza máxima, las diferencias fueron significativas ($P<0.05$) solo en las muestras presurizadas al final del período de almacenamiento a 8 ºC. El tiempo de almacenamiento en refrigeración afectó ($P<0.001$) a ambas propiedades de textura, detectándose valores menores al final del almacenamiento, tanto en el jamón no presurizado como en el tratado por altas presiones.

Los tratamientos de presurización a 400 y 500 MPa durante 10 min resultaron efectivos en la inactivación de *E. coli* O157:H7 inoculado en jamón curado loncheado, aunque las reducciones alcanzadas fueron pequeñas, 0.25 y 1.28 unidades logarítmicas por el efecto inmediato de la presurización. La aplicación individual de pediocina o nisina no afectó a la supervivencia del patógeno. En el presente trabajo no se detectó un aumento de la actividad antimicrobiana con la combinación de la pediocina con los tratamientos de alta presión, a diferencia de lo que ocurrió con la nisina. La inactivación alcanzada por la combinación de nisina con la presurización fue ligeramente superior a la inactivación obtenida individualmente, detectándose un efecto sinérgico que con el tratamiento de 500 MPa combinado con nisina se mantuvo durante 60 d a 8 ºC. Las células de *E. coli* dañadas por la presurización no

fueron capaces de recuperarse durante el período de refrigeración investigado. En las muestras no presurizadas, los recuentos de *E. coli* disminuyeron 0.43 unidades logarítmicas durante 60 d a 8 ºC, indicando la capacidad de supervivencia del patógeno en jamón curado durante un período de almacenamiento prolongado.

Respecto a los cambios en el color del jamón sometido a los distintos tratamientos combinados, cuando se investigó nisina o pediocina con presurizaciones de 500 MPa durante 10 min se observó una disminución de *L**. La luminosidad tendía a disminuir también en el jamón control y en el tratado durante el período de refrigeración, siendo los cambios inducidos por el almacenamiento a 8 ºC, como en el caso de los tratamientos individuales de alta presión, mayores que los originados por la presurización. Los valores de *a** resultaron ligeramente afectados por los tratamientos, tendiendo a disminuir con la refrigeración. Los valores de *b** no cambiaron significativamente en el jamón presurizado o tratado con las bacteriocinas. En la mayoría de los casos, los valores más bajos de *b** se obtuvieron después de 60 d de almacenamiento en refrigeración. En general, los cambios originados en las propiedades del color del jamón curado sometido a tratamientos de 400 o 500 MPa durante 10 min, individualmente o en combinación con los bioconservantes fueron bajos, observándose como los valores de los tres parámetros *L**, *a** y *b** tendían a disminuir durante el almacenamiento en refrigeración.

En jamón presurizado a 500 MPa durante 10 min, o con pediocina o nisina aplicadas individualmente, se registraron valores significativamente ($P<0.05$) menores de la fuerza al corte 1 d después de los tratamientos. Los valores de este parámetro tendían a aumentar durante la refrigeración, aunque las variaciones no mostraron una tendencia clara. Respecto a la fuerza máxima, se detectaron valores mayores ($P<0.05$) en las muestras tratadas con nisina o con la combinación de 400 MPa y nisina o pediocina, sin una tendencia clara durante el período de refrigeración.

Otra combinación investigada en la presente tesis doctoral fue la de APH a 450 MPa durante 10 min, el SLP o LF frente a *L. monocytogenes* y *S*. Enteritidis inoculados en jamón curado loncheado, almacenado durante 60 d a 8 ºC. *L. monocytogenes* mostró mayor resistencia al tratamiento de APH, alcanzándose reducciones de 0.58 unidades logarítmicas después de la presurización. Sin embargo, las reducciones de *S*. Enteritidis fueron superiores, mostrando la menor barotolerancia experimentada en general por los Gram-negativos. La aplicación individual de los dos bioconservantes ensayados, LF y el SLP, no afectó a los recuentos de los patógenos. Cuando se aplicaron tratamientos combinados de alta presión y LF o el SLP en jamón curado loncheado, se observó un efecto antimicrobiano sinérgico sobre *S*. Enteritidis, alcanzándose reducciones logarítmicas 1.45 y 2.09 mayores que la suma de la

inactivación obtenida con cada tratamiento individual. Este efecto bactericida aumentó a partir de los días 1 y 15 de almacenamiento a 8 ºC, de forma que el patógeno solo fue detectado mediante el enriquecimiento de las muestras. Frente a *L. monocytogenes* se observó un ligero efecto antimicrobiano sinérgico con la combinación de la alta presión y el SLP al final del período de almacenamiento. Los recuentos de ambos patógenos también disminuyeron en las muestras no tratadas durante el almacenamiento a 8 ºC, aunque *S*. Enteritidis resultó más afectado por las condiciones del jamón curado que *L. monocytogenes*, mostrando este último mayor capacidad para sobrevivir en jamón curado durante un almacenamiento prolongado.

Los niveles de viables totales en el jamón curado fueron menores en las muestras presurizadas y en las que se aplicaron tratamientos combinados que en las muestras control y en las que contenían LF y el SLP individualmente. Al final del período de almacenamiento se observó un aumento de viables totales en todas las muestras, aunque en las tratadas con la combinación de altas presiones y el SLP se registró un efecto antimicrobiano sinérgico de 2.19 unidades logarítmicas.

En el jamón curado tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF, o sus combinaciones no se observaron diferencias significativas en el valor de L^*, excepto en las muestras presurizadas y combinadas con el SLP, en las que se observó un aumento ($P<0.05$) de este parámetro. Sin embargo, este cambio se atenuó al final del período de refrigeración a 8 ºC. No se observaron diferencias significativas de a^*, tras la aplicación de los tratamientos, mientras que sus valores disminuyeron en las muestras tratadas con presión aplicada individualmente o en combinación, al final del almacenamiento. Por otro lado, los antimicrobianos aplicados individualmente no afectaron a los valores de a^*. En cuanto a la tendencia al amarillo, no se observaron diferencias significativas de b^* tras la aplicación de los tratamientos. Sin embargo, a los 60 d de refrigeración a 8 ºC se observó un aumento ($P<0.05$) de este parámetro en el jamón tratado con LF y una disminución ($P<0.05$) en el presurizado y combinado con el SLP. En el presente trabajo, los parámetros del color no fueron afectados por el SLP tras el tratamiento, demostrando que la generación *in situ* de H_2O_2 por la glucosa oxidasa (GOX) y la glucosa añadidas no afectaría negativamente al color del jamón curado.

En el jamón tratado con altas presiones a 450 MPa durante 10 min, el SLP, LF o sus combinaciones no se observaron diferencias significativas en los valores de la fuerza al corte ni de la fuerza máxima tras los tratamientos, excepto en el jamón tratado con LF, en el que se registró un aumento ($P<0.05$) de la fuerza máxima. Después de 60 d en refrigeración a 8 ºC, la fuerza máxima disminuyó ($P<0.05$) en el jamón

tratado con LF o en combinación con el tratamiento de alta presión y la fuerza al corte experimentó un aumento (P<0.05) con el SLP combinado con la presurización. No obstante, al final del período investigado no se registraron diferencias significativas entre el jamón sometido a los diferentes tratamientos y el control en ninguna de las propiedades de textura estudiadas.

Los tratamientos de presurización aumentaron ligeramente el pH del jamón curado, aunque las diferencias en las muestras tendieron a atenuarse al final del período de refrigeración. Los valores de a_w aumentaron en el jamón presurizado, aunque las diferencias no fueron suficientes para comprometer la seguridad microbiológica del producto.

En el carpaccio de ternera inoculado con *S*. Enteritidis se ensayaron tratamientos de 450 MPa durante 5, 10 y 15 min y el producto se mantuvo durante 30 d a 8 ºC. Inmediatamente después de la aplicación de 450 MPa durante 5 y 10 min se alcanzaron reducciones de 3.68 y 5.94 unidades logarítmicas, mientras que *Salmonella* solo se detectó por enriquecimiento de las muestras tratadas a 450 MPa durante 15 min. Los recuentos del patógeno disminuyeron únicamente 0.26 unidades logarítmicas en las muestras no presurizadas tras los 30 d de almacenamiento a 8 ºC, indicando la capacidad de *Salmonella* de sobrevivir en este producto cárnico. Durante la refrigeración, las reducciones con el tratamiento menos intenso fueron de 1.33 unidades logarítmicas y la presencia de *Salmonella* en 10 g de producto solo fue detectada tras el enriquecimiento de las muestras con los tratamientos de presurización más intensos, indicando que el patógeno no fue capaz de recuperarse bajo las condiciones ensayadas.

Respecto a los microorganismos viables totales, se comprobó un aumento en los niveles en el carpaccio control durante el almacenamiento a 8 ºC, mientras que la presurización mantuvo los recuentos en valores significativamente menores (P<0.05) que los detectados en el carpaccio control.

Los tratamientos de altas presiones a 450 MPa durante 5, 10 o 15 min aumentaron la luminosidad (L^*) del carpaccio. Este efecto se observó durante el período de refrigeración investigado, con una tendencia creciente en las muestras presurizadas durante 15 min. Los valores de la tendencia al rojo (a^*) disminuyeron en el carpaccio tratado por alta presión (P<0.05) tras la aplicación de los tratamientos. Los valores más bajos se detectaron al final del período de refrigeración en las muestras presurizadas a 450 MPa durante 15 min. Los valores de la tendencia al amarillo (b^*) disminuyeron (P<0.05) en el carpaccio presurizado en comparación con las muestras control, aunque estas diferencias se atenuaron durante el almacenamiento a 8 ºC.

En relación a la textura, la fuerza al corte (N) disminuyó ($P<0.05$) en el carpaccio presurizado a 450 MPa durante 5, 10 y 15 min, inmediatamente después del tratamiento, aunque las diferencias fueron menores a mayor tiempo de presurización. Por otro lado, no se detectaron diferencias significativas en la fuerza máxima después de los tratamientos entre el carpaccio tratado por altas presiones y el no presurizado. Durante la conservación a 8 ºC ambos parámetros incrementaron su valor en el carpaccio presurizado.

Con objeto de inactivar *L. monocytogenes*, *S*. Enteritidis y *E. coli* O157:H7 inoculados en carpaccio de ternera y observar el comportamiento de los patógenos en condiciones de abuso de temperatura a 8 ºC y a temperatura ambiente de 22 ºC durante 7 d, se aplicaron tratamientos individuales de 450 MPa durante 5 min, el SLP, LF activada (ALF) y sus combinaciones. Los patógenos no fueron capaces de crecer en el carpaccio no tratado durante el período investigado. Los niveles de *S*. Enteritidis disminuyeron durante el período de almacenamiento investigado.

Las altas presiones ejercieron un efecto bactericida sobre los tres patógenos, aunque *S*. Enteritidis mostró la mayor sensibilidad al tratamiento de presurización, seguido de *E. coli* y *L. monocytogenes*.

No se detectó actividad antimicrobiana del SLP ni de la ALF, aplicados individualmente en el carpaccio de ternera. La combinación de las altas presiones y el SLP produjo un efecto bactericida sinérgico frente a *S*. Enteritidis y *E. coli* O157:H7 en carpaccio de ternera. En el caso de *L. monocytogenes* y *E. coli* O157:H7, las reducciones detectadas a los 7 d de almacenamiento en las muestras tratadas con el SLP en combinación con altas presiones fueron mayores a 22 ºC que a 8 ºC, al contrario de lo que ocurrió con *Salmonella*. El tratamiento combinado entre ALF y altas presiones no aportó ninguna mejora al potencial antimicrobiano de las altas presiones frente a los tres patógenos estudiados.

En las muestras de carpaccio tratadas con el SLP, ALF o altas presiones a 450 MPa durante 5 min, los niveles de microorganismos viables totales se redujeron 3.28, 3.24 y 3.55 unidades logarítmicas, respectivamente, tras la aplicación de los tratamientos. Con la combinación de la presurización y ALF o el SLP, las reducciones registradas fueron de 3.69 y 3.82 unidades logarítmicas. Aunque durante el almacenamiento a 8 ºC, los niveles de microorganismos viables totales aumentaron en el carpaccio control y en el tratado, se mantuvieron en valores más bajos en las muestras tratadas hasta el final del período investigado.

En el carpaccio tratado a 450 MPa durante 5 min en combinación con el SLP o ALF se observó un aumento (P<0.05) del valor de L^*, inmediatamente después de la presurización. Se observó una disminución de L^* en las muestras tratadas con ALF inmediatamente después de la presurización, pero no se observó efecto del SLP sobre la luminosidad. Al final del período de refrigeración a 8 ºC, los valores de L^* no cambiaron significativamente, excepto en las muestras tratadas con ALF aplicada individualmente o en combinación con las altas presiones, registrándose un aumento y una disminución en los valores de este parámetro, respectivamente. Inmediatamente después de la presurización, la aplicación individual del SLP no afectó a la tendencia al rojo (a^*) ni al amarillo (b^*) del carpaccio de ternera. Después de 30 d a 8 ºC se observó un aumento de a^* en las muestras presurizadas y una disminución en las muestras tratadas con ALF. Respecto a la tendencia al amarillo, se observó una disminución de b^* (P<0.05) en las muestras no tratadas, en las tratadas con ALF y en aquellas tratadas con el SLP en combinación con las altas presiones al final del período investigado.

La fuerza al corte no se vio afectada por los tratamientos de alta presión a 450 MPa, el SLP, ALF, o sus combinaciones, excepto por el tratamiento de presurización combinado con el SLP que originó un aumento (P<0.05) de este parámetro, inmediatamente después de la presurización. Después de 30 d a 8 ºC, los valores de la fuerza al corte aumentaron (P<0.05) con la aplicación individual de los antimicrobianos y disminuyeron con el tratamiento combinado entre altas presiones y el SLP, en comparación con el tiempo 0 h. La dureza del carpaccio de ternera determinada como fuerza máxima no se vio afectada por los tratamientos. Después de 30 d a 8 ºC, se observó un aumento (P<0.05) de esta propiedad en las muestras tratadas con el SLP individualmente.

La siguiente combinación ensayada en este producto frente a *L. monocytogenes*, *S*. Enteritidis y *E. coli* O157:H7 consistió en aplicar tratamientos de 450 MPa durante 5 min con las bacteriocinas enterocinas A y B, pediocina PA-1, nisina Z, y nisina y pediocina comerciales. Las bacteriocinas aplicadas individualmente produjeron reducciones entre 0.1 y 1.5 unidades logarítmicas en los recuentos de *L. monocytogenes*, siendo la pediocina comercial la bacteriocina más efectiva frente al patógeno. Por otro lado, la nisina Z fue la bacteriocina menos efectiva frente a *Listeria*. En el caso de *S*. Enteritidis y *E. coli* O157:H7, las bacteriocinas aplicadas individualmente no mostraron actividad antimicrobiana.

El tratamiento de presurización de 450 MPa durante 5 min fue más efectivo frente a *S*. Enteritidis, con reducciones superiores a 4 unidades logarítmicas, seguido de *E. coli* O157:H7 y *L. monocytogenes*, que mostraron mayor barotolerancia.

Inmediatamente después de la presurización se detectó un efecto antimicrobiano ligeramente sinérgico frente a *Listeria* con los tratamientos combinados de enterocinas A y B o nisina comercial con las altas presiones. Sin embargo, con la combinación de pediocina PA-1, nisina Z o pediocina comercial el efecto antimicrobiano solo fue aditivo. En el caso de *S*. Enteritidis, el tratamiento combinado entre las bacteriocinas y las altas presiones no aportó ninguna mejora al potencial antimicrobiano de la presurización. En el caso de *E. coli*, se observó un efecto antimicrobiano ligeramente sinérgico con la mayoría de los tratamientos combinados, excepto con la pediocina PA-1 con la que se registró un efecto aditivo. Al final del período de almacenamiento, el efecto antimicrobiano sobre *Listeria* fue aditivo con la combinación de las enterocinas A y B o la nisina comercial y las altas presiones, y ligeramente sinérgico con la nisina Z o la pediocina comercial con la presurización. En el caso de *Salmonella*, se detectó un efecto antimicrobiano aditivo entre las altas presiones y las enterocinas A y B o la pediocina PA-1. En el caso de *E. coli*, se observó un efecto antimicrobiano sinérgico con los tratamientos combinados entre las altas presiones y las enterocinas A y B, la pediocina PA-1 o la nisina comercial, detectándose índices de inactivación 2 unidades logarítmicas superiores a los alcanzados con la suma de los tratamientos individuales. Durante el almacenamiento a 8 ºC, ninguno de los tres patógenos estudiados creció en carpaccio no tratado.

La presurización redujo los niveles de viables totales en 2.14 unidades logarítmicas y la aplicación individual de las enterocinas A y B, pediocina PA-1 o nisina Z dio lugar a reducciones de 0.36, 0.55 y 0.28 unidades logarítmicas, respectivamente. La bacteriocina más efectiva fue la nisina comercial, que disminuyó los niveles de viables totales 1.18 unidades logarítmicas. El efecto de las altas presiones combinadas con la pediocina comercial produjo un efecto antimicrobiano sinérgico, con reducciones 0.88 unidades logarítmicas mayores que la suma de la inactivación obtenida con cada tratamiento individual. Durante el almacenamiento en refrigeración a 8 ºC, los niveles de viables totales aumentaron 0.94 unidades logarítmicas en las muestras de carpaccio no tratadas. En las muestras de carpaccio tratadas con nisina o pediocina comercial en combinación con las altas presiones, se observó un ligero efecto antimicrobiano sinérgico al final del período investigado. En el resto se detectó una recuperación de los viables totales.

La adición de bacteriocinas al carpaccio de ternera no afectó a los valores de L^*. Sin embargo, se observó un aumento en los valores de este parámetro con todos los tratamientos combinados de bacteriocinas y altas presiones a 450 MPa durante 5 min, aunque fue significativamente ($P<0.05$) mayor en las muestras presurizadas en combinación con la pediocina PA-1 o con la nisina comercial, respectivamente.

Después de 30 d a 8 ºC, los valores de *L** disminuyeron ($P<0.05$) en las muestras tratadas con enterocinas A y B y aumentaron ($P<0.05$) en las muestras tratadas con pediocina comercial.

Inmediatamente después de la presurización, la tendencia al rojo (*a**) disminuyó ($P<0.05$) con la presurización aplicada individualmente o en combinación con las bacteriocinas. Solo con nisina Z se observó la disminución de los valores de *a**. Sin embargo, las diferencias en *a** tendían a atenuarse con todos los tratamientos al final del almacenamiento.

En cuanto a la tendencia al amarillo (*b**) se observó una disminución de los valores en las muestras presurizadas o en aquellas combinadas con las bacteriocinas, aunque esta disminución fue significativa ($P<0.05$) solo con pediocina PA-1 o pediocina comercial combinadas con altas presiones. Después de 30 d a 8 ºC, las diferencias en los valores de *b** tendían a atenuarse. De los resultados obtenidos en el presente trabajo se desprende que los cambios detectados en el carpaccio de ternera fueron principalmente debidos al efecto de las altas presiones y al tiempo de almacenamiento, sin que las bacteriocinas tuvieran una influencia reseñable en las características del producto.

En el carpaccio sometido a altas presiones en combinación con bacteriocinas no se observaron diferencias significativas en la fuerza al corte inmediatamente después de la presurización. Después de 30 d a 8 ºC, este parámetro aumentó ($P<0.05$) en las muestras tratadas con enterocinas A y B, pediocina PA-1, nisina Z, pediocina comercial, y en las muestras presurizadas combinadas con nisina Z o nisina comercial. Respecto a la dureza, solo aumentó significativamente ($P<0.05$) en las muestras tratadas con pediocina comercial individualmente o en combinación con la alta presión, inmediatamente después de la presurización. Al final del período investigado, este parámetro aumentó ($P<0.05$) en las muestras no tratadas y en las tratadas con enterocinas A y B individualmente o combinadas con altas presiones.

Los tratamientos de altas presiones aumentaron ligeramente los valores de pH del carpaccio de ternera y la aplicación individual de pediocina PA-1, nisina comercial o la combinación de 450 MPa durante 5 min y nisina comercial produjo una disminución del mismo, inmediatamente después de la presurización. Después de 30 d a 8 ºC, el pH disminuyó en todas las muestras. Los valores de a_w aumentaron en el carpaccio tratado con altas presiones, individualmente o en combinación con el SLP o con ALF. También se registró un aumento en los valores de a_w en el carpaccio tratado con enterocinas A y B, pediocina PA-1, nisina Z o con la combinación de 450 MPa durante 5 min y enterocinas A y B o nisina comercial.

Por último, se ha investigado el efecto de tratamientos combinados de nitrito sódico y altas presiones en la inactivación de *E. coli* y *L. monocytogenes* en sistemas tampón considerando la posibilidad de su posterior aplicación en productos cárnicos. Se emplearon concentraciones de 0.25, 0.50, 0.75, 1.0, 2.0 y 3.0 mM de nitrito sódico acidificado a pH 4.0. Las células de ambos patógenos fueron sensibles al nitrito sódico. La exposición de *E. coli* a 1.0, 2.0 y 3.0 mM de nitrito sódico acidificado causó una reducción inmediata en los recuentos de viables inferior a una unidad logarítmica pero después del almacenamiento a 4 ºC durante 24 h, las reducciones aumentaron 1.10, 2.60 y 5 unidades logarítmicas, respectivamente. La presencia de 0.25, 0.50 y 0.75 mM de nitrito sódico acidificado causó reducciones de *L. monocytogenes* de 0.90, 2.30 y 3.60 unidades logarítmicas, respectivamente, después de 24 h a 4 ºC.

La aplicación del tratamiento de 300 MPa durante 8 min causó una reducción de 1.40 unidades logarítmicas de *E. coli*. Cuando la presurización se combinó con 1.0 ó 2.0 mM de nitrito sódico, el número de viables disminuyó 2.40 y 4.90 unidades logarítmicas, respectivamente, mientras que con 3.0 mM de nitrito sódico, los niveles de *E. coli* se situaron por debajo del límite de detección (<2.4 unidades logarítmicas), indicando reducciones de más de 6 unidades logarítmicas. El grado de inactivación, 24 h más tarde, fue superior a 6 unidades logarítmicas con todos los tratamientos combinados. El tratamiento de presión de 225 MPa durante 8 min causó una reducción de una unidad logarítmica en la viabilidad de *L. monocytogenes*. Con los tratamientos de alta presión en combinación con 0.25, 0.50 y 0.75 mM de nitrito sódico, se obtuvieron reducciones de 1.50, 2.70 y 4.20 unidades logarítmicas. Veinticuatro horas más tarde de la aplicación de los tratamientos se observó un aumento (P<0.001) de la inactivación, alcanzando reducciones de 3.40, 6.10 y 6.10 unidades logarítmicas, respectivamente.

El efecto antimicrobiano de la combinación de nitrito sódico y las altas presiones resultó sinérgico sobre *E. coli* y *L. monocytogenes.* Se estudió el efecto del pH sobre la inactivación de *E. coli* por altas presiones combinadas con 2.0 mM de nitrito sódico acidificado a pH 4.0, detectándose reducciones de aproximadamente 4 unidades logarítmicas inmediatamente después de la presurización, pero no se observaron efectos antimicrobianos a pH 5.0 ni a pH 7.0. No obstante, a pH 4.5 se observó un efecto antimicrobiano sinérgico ligero con la combinación de las altas presiones y 3.0 mM de nitrito sódico. Bajo estas condiciones, el tratamiento combinado causó una reducción de 1.40 unidades logarítmicas comparado con las 0.23 unidades logarítmicas obtenidas con la suma de los tratamientos por separado. Se demuestra así, la dependencia del pH con el efecto bactericida del nitrito.

Con objeto de profundizar en el efecto combinado, se investigó con los mutantes *hmp*A y *nor*V, carentes de las enzimas protectoras dioxigenasa del óxido nítrico y de la reductasa del óxido nítrico, respectivamente (Gardner *et al*., 2002; Poole, 2005) de *E. coli* BW25113. Estos mutantes fueron significativamente más sensibles al nitrito acidificado que la cepa parental, e incluso más sensibles al tratamiento de alta presión aplicado individualmente. Además, las diferencias en los efectos antimicrobianos sinérgicos detectadas entre las cepas no fueron estadísticamente significativas.

CONCLUSIONES

Los tratamientos de alta presión a 500 y 600 MPa durante 5 min resultaron eficaces para inactivar *S*. Enteritidis en jamón curado loncheado. No se registró recuperación del patógeno durante el almacenamiento posterior en condiciones de abuso de temperatura.

La combinación de 400 o 500 MPa durante 10 min con nisina comercial mostró un efecto antimicrobiano sinérgico sobre *E. coli* O157:H7 en jamón curado loncheado. Este efecto se mantuvo con el tratamiento combinado de 500 MPa y nisina durante el almacenamiento del producto.

La combinación de 450 MPa durante 10 min con el sistema lactoperoxidasa o lactoferrina produjo un efecto antimicrobiano sinérgico frente a *S*. Enteritidis en jamón curado loncheado. El efecto alcanzado con la combinación de 450 MPa y el sistema lactoperoxidasa fue sinérgico al final de la refrigeración frente a *L. monocytogenes*.

Los cambios en la textura y color del jamón curado fueron poco acusados. La presurización produjo un ligero aumento de la opacidad y una menor tendencia al rojo, así como alguna modificación de la textura. Dichos cambios tendieron a atenuarse durante el almacenamiento.

Los tratamientos de 450 MPa durante 5, 10 y 15 min inactivaron *S*. Enteritidis en carpaccio de ternera refrigerado. La reducción de *Salmonella* alcanzó 5 unidades logarítmicas con el tratamiento menos intenso.

El tratamiento de 450 MPa durante 5 min combinado con el sistema lactoperoxidasa o lactoferrina activada resultaron eficaces en la inactivación de *S*. Enteritidis y *E. coli* O157:H7 en carpaccio de ternera. La combinación de 450 MPa y el sistema lactoperoxidasa mostró un efecto antimicrobiano sinérgico frente a *S*. Enteritidis y *E. coli* O157:H7.

La presurización del carpaccio de ternera a 450 MPa durante 5 min o su combinación con las enterocinas A y B, pediocina PA-1, nisina Z, nisina o pediocina comercial inactivaron *S*. Enteritidis y *E. coli* O157:H7. En el caso de *E. coli* O157:H7, el efecto antimicrobiano de la alta presión combinada con las enterocinas A y B, pediocina PA-1 o nisina comercial fue sinérgico.

Los tratamientos modificaron ligeramente el color y la textura del carpaccio de ternera con un ligero aumento de la opacidad y una menor tendencia al rojo. La dureza aumentó al final del período de refrigeración con los tratamientos más intensos.

Se comprobó el efecto bactericida del nitrito aplicado individualmente o en combinación con las altas presiones sobre *E. coli* y *L. monocytogenes* en estudios *in vitro.* El efecto antimicrobiano combinado fue sinérgico a pH 4.0 frente a ambos microorganismos.

Los mutantes *hmp*A y *nor*V de *E. coli* BW25113 fueron más sensibles al nitrito acidificado y al tratamiento de alta presión que la cepa parental apuntando a la participación del óxido nítrico en el efecto bactericida.

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9. Extended abstract[1]

[1]This extended abstract is included in fulfilment of the directives of the regulation of development of the Real Decreto 99/2011, de 28 de enero, which regulates the studies of doctorate at the Universidad Complutense de Madrid (UCM) (BOUC nº14, de 21 de diciembre de 2012) and in agreement with the specifications established by the Commission of Doctorate of the UCM.

INTRODUCTION

The lifestyle of the current society in developed countries has given rise to changes in the food habits of consumers. The growing demand for less treated but more stable and safer food, with a longer shelf-life, easier to prepare, nutritious and processed without chemical preservatives can entail a lack of intrinsic preservation and protection throughout processing.

Processed foodstuffs have further been transformed by the food industry in ready-to-eat (RTE) food in order to satisfy consumers´demand. According to Regulation 2073/2005, RTE food can be defined as food intended by the producer or the manufacturer to be consumed without the need to be cooked or otherwise processed in order to eliminate or reduce to an acceptable level microorganisms of concern thus ensuring its safety and compliance (EC, 2005). In most of the cases, RTE foods are either vacuum-packaged or modified atmosphere packaged (MAP) and kept at refrigeration temperatures in order to extend their shelf-life.

RTE food processing that involves a size reduction, slicing, cutting into pieces, as well as measuring out or packaging. All these steps increase the risks of an accidental contamination, which can be enhanced in abuse or uncontrolled temperature conditions (Cava *et al*., 2009; Garrido *et al*., 2010).

RTE foods can be contaminated during their manufacture/processing with pathogen microorganisms from contaminated environments, equipment or food handlers. The cold chain should, therefore, be kept and food should be prepared according to related microbiological criteria. Several *Escherichia coli* serovars (including O157:H7), *Salmonella* spp., *Campylobacter jejuni*, *Listeria monocytogenes*, *Yersinia enterocolitica* and *Staphylococcus aureus* are the pathogen microrganisms most frequently associated with contaminated food. Among them, *L. monocytogenes* and *Salmonella* spp. can be considered ubiquitous thus being detected in a wide variety of foodstuffs. *L. monocytogenes* is the foodborne pathogen of major concern, since it can grow at refrigeration temperatures without overtake 4 ºC. *E. coli* O157:H7 has a very low infective dose and cause a very severe illness. *S. aureus* is an opportunist microorganism, whose growth in foodstuffs kept at refrigeration temperature depends on storage temperature conditions.

The implementation of procedures based on hazard analysis and critical control point (HACCP) principles must guarantee food safety. However, these may not be enough. The application of non-thermal technologies such as high hydrostatic pressure (HHP), pulsed light technology, radiations, etc., and some biological agents (individually applied or in combination with some of the cited technologies, such as organic acids,

bacteriocins, and other antimicrobial agents are gaining ground like effective tools to ensure the higienization of RTE foods.

According to microbiological criteria for different foodborne pathogen, US and EC food regulations require *Salmonella* and *E. coli* to be absent in 25 g. However, the guideline for *L. monocytogenes* in RTE foods differs among countries. In this respect, the United States Department of Agriculture (USDA) has established a "zero tolerance" policy for *L. monocytogenes*, whereas differences within recipient countries and food category can be found in the EU regulations (CE, 2007). For RTE foods showing any of the following properties: pH≤4.4, a_w≤0.92, pH≤5.0 and a_w≤0.94, and for those with a shelf-life below 5 days, the EU regulation permit counts of up to 100 cfu/g when *Listeria* does not multiply during storage of foods for healthy population.

In the EU, and therefore in Spain, the current microbiological criteria are governed by the 2073/2005 regulation (CE, 2005) and its subsequent modifications: the 1441/2007 (CE, 2007), the 365/2010 (UE, 2010) and the 1086/2011 (UE, 2011) regulations.

According to the recently published European Food Safety Authority (EFSA) report, the prevalence of both *Salmonella* and *L. monocytogenes* has decreased over the past years, whereas verocytotoxin-producing *E. coli* (VTEC) infections have risen from 2008 (EFSA, 2013) on.

Traditional technologies (such as the temperature control, a_w or redox potential), combined with other preservation technologies (such as MAP, bacteriocins, organic acids, bioprotective cultures, HHP, etc.) could establish a series of preservative factors (the so-called hurdles) that spoilage and pathogen microorganisms should not be able to overcome, without exerting a detrimental effect on non-spoilage microorganisms. Numerous barriers or hurdles can be used for a given foodstuff (Leistner, 2000) in order to control microbial growth and/or development, and these might exert an additive or synergistic effect.

In the present study HHP has been chosen as a non-thermal preservation technology aimed to enhance microbial safety and food shelf-life and it has been combined with several biopreservation strategies, namely bacteriocins, lactoperoxidase system (LPS) and lactoferrin (LF), in an attempt to potenciate possible synergistic effects and thus to minimize the intensity of HHP on two RTE meat products: sliced dry-cured ham and beef carpaccio. Furthermore, the effect of the above mentioned

technologies on the physicochemical and rheological characteristics as well as on the color of both meat products has been assessed.

As a consequence of the positive consumer´s perception of HHP, the food industry has implemented this technology more extendedly than other emerging technologies. Regarding the meat sector, treatment conditions generally comprise pressure levels of 400-600 MPa, which are applied for 3-7 min at ambient temperature. These conditions usually entail inactivation levels of approximately 4 log units for most of the spoilage and pathogens microorganisms (Bajovic *et al*., 2012).

In general, HHP-induced changes in color of meat and meat products depend on different factors, such as the myoglobin content, being these changes more pronounced in fresh red meat than in white meat and cured meat products. The negative effect of HPP on color may be diminished by optimizing pressurization conditions, curing agents, oxygen availability or pH. Concerning texture, the influence of HHP greatly depends on the meat rigor-mortis, pressure levels and applied temperature (Ma *et al*., 2004) as well as on the time of exposure (Sun y Holley, 2010).

Bacteriocins are ribosomally synthesised polypeptides or proteins with molecular weights comprised between 900 and 5800 Daltons (Da), that in their mature form, exert some antimicrobial effect against bacteria closely related to bacteriocin-producing bacteria. Bacteriocins are produced by both Gram-negative and Gram-positive bacteria. Bacteriocin production may provide a mechanism by which producers can gain a competitive advantage over neighbouring sensitive strains. Generally, bacteriocins form pores in the cell membrane, thus depleting the proton gradient force across them, but they can also inhibit protein synthesis, messenger-RNA production and DNA duplication.

In Food Microbiology, bacteriocins produced by lactic acid bacteria strains are of utmost interest since they usually exert their bactericidal effect against different spoilage and foodborne Gram-positive bacteria. Gram-negative bacteria are resistant to lactic acid bacteria-produced bacteriocins. Nevertheless, under chemical or physical stress, bacteriocins can alter the lypopolysaccharide structure of the external membrane and exert their bactericidal action against different Gram-negative bacteria of especial interest for the food industry (Fang y Tsai, 2003; Kalchayanand *et al*., 1992, 1998; Stevens *et al*., 1991). It should be noted that *in vitro* bacteriocin production (that is, in laboratory media) does not entail any antibacterial effect in real food systems. Meat and meat products are complex matrixes in which bacteriocin activities can be partially or totally decreased their activity. Furthermore,

bacteriocins may be modified during food processing or throughout storage as a consequence of either their interaction with food components or their inactivation by proteases thus leading to solubility or charge changes (Aasen *et al*., 2003).

Nisin is produced by certain *Lactococcus lactis* and *Streptococcus* sp. strains. Different nisin forms have been reported, with nisins A, Z, Q and F being produced by *L. lactis* and nisin U and U2 being produced by *S. uberis* 42 and *S. agalactiae* D536. These nisin forms differ from each other in some of their amino acid residues. Nisin is the only bacteriocin having the GRAS (Generally Regarded As Safe) status by the USA Food and Drug Agency (FDA) and, therefore, it is commercially available (O´Keeffe y Farrell, 2000) in more than 50 countries, including USA and some Member States of the European Union. Nisin is included as additive E234 in the 95/2/EC directive on authorised food additives other than colors and sweeteners without known adverse effects. Nisin is also a representative of the class Ia of the Klaenhammer´s classification (Klaenhammer, 1993). It prevents spores of Gram-positive bacteria such as *Clostridium* and *Bacillus* from germinate (Savadogo *et al*., 2006) and also shows activity against Gram-positive bacteria such as *Staphylococcus*, *Listeria*, *Enterococcus*, etc.

Pediocin PA-1 is produced by *Pediococcus acidilactici*, although it can also be produced by other *Pediococcus* species and even by certain lactic acid bacteria (Miller *et al*., 2005). It belongs to the class IIa of Klaenhammer´s classification (Klaenhammer, 1993) and shows activity against some species of *Lactococcus*, *Lactobacillus*, *Carnobacterium*, *Enterococcus*, *Bacillus*, *Brochothrix*, *Clostridium*, *Listeria* and *Staphylococcus*, although the sensitivity of these depends on the species and even on the strain. Pediocin PA-1 shows inhibitory activity against *L. monocytogenes* (Nielsen *et al*., 1990).

Enterocins are bacteriocins produced by bacteria from the *Enterococcus* genera. Amongst enterocins that have been previously described enterocins A and B, AS-48, I, L50A and L50B, CCM 4231, 13, 416K1, etc. are of special interest (Khan *et al*., 2010). Enterocins A and B, that belong to class IIa (Klaenhammer, 1993), are produced by certain *Enterococcus faecium* strains, the former being isolated from the *E. faecium* CTC 492 strain and enterocin B being isolated from *E. faecium* T136. Both enterocin A and B act synergistically with each other and are active against a wide spectrum of Gram-positive bacteria, species of *Clostridium*, *Propionibacterium*, *Listeria*, *Staphylococcus* and most of the lactic acid bacteria being included. Different studies have demonstrated that enterocins A and B are active against *L. monocytogenes*, *Salmonella* and *S. aureus* that had been previously inoculated in

meat products (Garriga *et al*., 2002). Both enterocins, in addition, provided extra protection after a cold chain break during storage (Marcos *et al*., 2008).

The LPS belongs to the defence mechanisms of the mammalian innate immune response. It can be found in different exocrine secretions and it consists of three components, namely the lactoperoxidase enzyme (LPX), the thiocyanate ion (SCN-) and hydrogen peroxide (H_2O_2), the two latter components being necessary for the LPS activation. The LPX enzyme, with the help of H_2O_2, catalyzes the oxidation of SCN- yielding intermediate active compounds that show antimicrobial properties. These intermediates exert their action towards enzymes´ sulfhydryl groups and some other proteins located in the bacterial membranes. The activation of the LPS requires either an exogenous addition of H_2O_2 or its generation by the action of exogenous enzymes such as the glucose oxidase (GOX). The presence of GOX together with its substrate, allows the continuous production of H_2O_2, as required by the LPS (Seifu *et al*., 2005). The LPS-GOX system might be composed of the LPX and GOX enzymes, glucose and a source of either sodium of potassium isothiocyanate ions. The LPS action depends on several factors, such as the bacterial species, the medium pH, the incubation time, the temperature and the cell density (Björck *et al*., 1975).

LF is a glycoprotein with a molecular weight of approximately 80 KDa belonging to the transferring family. The LF capability of binding iron appears to be responsible for LF´s bacteriostatic effect whereas its bactericidal effect seems to be due to the ability of LF to bind to some components of bacterial walls, such as lipopolysaccharides in Gram-negative bacteria and teicoic or lipoteicoic acids in Gram-positive bacteria, or even due to its alteration capacity of the cited components. Hence, depolarization and disruption of the membrane integrity and the pH gradient occurs (Vorland *et al*., 1999). LF does also have derivatives, such as amidated lactoferrin (AMILF) and its purified (lactoferricine, LFC) and non-purified (PDLF) forms, both of them being pepsin-digested. These substances have been proposed, due to their wide antibacterial spectra, as natural preservatives for food (Naidu, 2002).

OBJECTIVES

The objectives of the present thesis are achieving the maximum microbiological safety of ready to eat (RTE) meat products by HHP treatments and biopreservation.

In this context, the following partial objectives were considered:

1.- To inactivate *L. monocytogenes*, *S. enterica* ser Enteritidis and *E. coli* O157:H7 in sliced dry-cured ham and beef carpaccio by means of high pressure treatments

applied alone or in combination with bacteriocins, the lactoperoxidase system or lactoferrin.

2.- To evaluate the changes in the physicochemical, rheological and color characteristics of these products as a consequence of the inactivation treatments.

3.- To investigate *in vitro* new combined treatments of sodium nitrite and high pressure to inactivate *L. monocytogenes* and *E. coli*.

RESULTS

High pressure treatments at 400, 500 and 600 MPa for 5 min were effective on the inactivation of *S. enterica* ser. Enteritidis inoculated on sliced dry-cured ham stored for 60 d at 8 ºC. The highest log unit reductions were achieved with the most intense treatments. Cells damaged by pressurization were not able to recover during storage, even under temperature abuse conditions. *Salmonell*a was not detected by direct count in dry-cured ham treated at 600 MPa from day 7 on, although after an enrichment step with 20 g of product its presence could be detected. *Salmonella* levels decreased gradually in non-treated samples during the investigated period, not being able to grow at 8 ºC because of the low a_w and high NaCl content in dry-cured ham.

Lipid oxidation increased with pressurization and storage and the content of free amino acids was similar in control and pressurized samples after 60 d of storage at 8 ºC. Regarding color, lightness (L^*) increased in dry-cured ham treated at 500 and 600 MPa for 5 min compared to non-treated samples and those pressurized at 400 MPa. The L^* values decreased ($P<0.001$) during refrigerated storage in both treated and control samples and changes observed by refrigeration time were higher than those induced by pressurization. Samples treated at 500 and 600 MPa showed lower a^* values than samples treated at 400 MPa or control. This difference was maintained for the whole 60 d period at 8 ºC with lower values at the end of storage. Yellowness values (b^*) were significantly ($P<0.001$) influenced by HHP. After 24 h, samples treated at 600 MPa exhibited significantly lower values compared with to the other pressurized and non-pressurized samples. Yellowness decreased during storage ($P<0.05$), with lower b^* values in dry-cured ham pressurized at 400 MPa and 600 MPa after 60 d at 8 ºC.

Regarding texture, pressurization reduced shear resistance in dry-cured ham treated at 500 or 600 MPa for 5 min when compared to non-pressurized samples and those treated at 400 MPa. Regarding maximum force, differences were only significant ($P<0.05$) in pressurized samples at the end of the 8 ºC storage. The storage time

affected (P<0.001) both texture properties, with lower values at the end of the storage period in both non-pressurized and pressurized dry-cured ham.

High pressure treatments at 400 and 500 MPa for 10 min were effective on the inactivation of *E. coli* O157:H7 inoculated on sliced dry-cured ham, although reduction values of only 0.25 and 1.28 log units were recorded immediately after pressurization. The application of commercial pediocin or nisin alone did not affect the survival of this pathogen. The antimicrobial activity did not increase when pediocin and pressurization were combined, different from nisin. However, when nisin was combined with HHP, an antimicrobial synergistic effect was detected. The inactivation of *E. coli* was slightly higher than the sum of those achieved with each single treatment. The synergy was maintained after 60 d at 8 ºC in samples treated at 500 MPa for 10 min with nisin. Damaged *E. coli* cells were not able to recover during the investigated period. In non-pressurized samples, *E. coli* counts only diminished 0.43 log units during the 60 d period at 8 ºC, indicating the ability of this pathogen to survive in dry-cured ham during a prolonged storage.

A decrease of L^* values was observed in dry-cured ham treated at 500 MPa for 10 min combined with nisin or pediocin. Lightness tended to decrease in control and treated samples during the refrigerated period, being the changes induced by storage at 8 ºC bigger than those induced by pressurization. The a^* values were slightly affected by treatments and tended to decrease with refrigeration. The b^* values did not significantly change in pressurized or bacteriocin-treated samples. In most cases, the lowest b^* values were obtained after 60 d of storage. In general, changes in the color properties of dry-cured ham treated at 400 or 500 MPa for 10 min, by itself or in combination with biopreservatives were low, and L^*, a^* and b^* values tended to decrease during refrigerated storage.

In dry cured ham treated at 500 MPa for 10 min, the application of pediocin or nisin, significantly lower (P<0.05) the shear force values after 1 d. However, they tended to increase during refrigeration, although variations did not show a clear tendency. Regarding maximum force, higher (P<0.05) values were detected in samples treated with nisin or with the combination of 400 MPa and nisin or pediocin, although no clear tendency was observed during refrigeration storage.

Another combination investigated in the present work was to apply 450 MPa for 10 min, and the LPS or LF against *L. monocytogenes* and *S*. Enteritidis inoculated on sliced dry-cured ham stored at 8 ºC for 60 d. *L. monocytogenes* was more resistant to high pressure treatment than *S*. Enteritidis. Reductions of only 0.58 log units after pressurization were recorded for *L. monocytogenes*. *S*. Enteritidis showed lower

barotolerance, as generally expected for Gram-negatives. Reductions of 2.22 log units were recorded for *S*. Enteritidis. Biopreservatives on their own did not affect pathogen counts. A synergistic antimicrobial effect in dry-cured ham was detected when high pressure and LF or LPS were combined, with reductions 1.45 and 2.09 higher than the sum of the inactivations obtained with each single treatment immediately after treatments. This bactericidal effect increased after 1 and 15 d of storage at 8 ºC and *Salmonella* was only detected after sample enrichment. A slightly synergistic antimicrobial effect against *L. monocytogenes* was observed when high pressure and the LPS were combined at the end of storage. Both pathogen counts also decreased in non-treated samples during the 8 ºC storage. *L. monocytogenes* showed higher ability to survive in dry-cured ham during a prolonged storage than *S*. Enteritidis.

Levels of total viable counts (TVC) were lower in pressurized samples and in those with combined treatments than in control samples and in those treated with LF or the LPS. An increase of TVC was observed in all samples at the end of storage, although a synergistic antimicrobial effect of 2.19 log units was detected when high pressure and the LPS were combined.

No significant differences in L^* values were observed in dry-cured ham treated at 450 MPa for 10 min, the LPS, LF, or their combinations, except in samples in which high pressure and the LPS were combined, although . However, this change was attenuated at the end of the 8 ºC storage. No significant differences in a^* values were observed after treatments. However, a decrease of this parameter was observed in pressurized or combined samples, at the end of the storage. Antimicrobials by themselves did not affect a^* values. No significant differences were detected in yellowness values (b^*) after treatments. However, an increase ($P<0.05$) of this parameter was observed in dry-cured ham treated with LF and a decrease ($P<0.05$) was detected in high pressure treated-samples with the LPS after 60 d of storage at 8 ºC. In the present work, the color of dry-cured ham was not affected by the LPS application, showing that the H_2O_2 *in situ* generation by glucose oxidase and glucose had no effect on this property.

No significant differences in shear force and maximum force were observed in dry-cured ham treated at 450 MPa for 10 min, the LPS, LF or their combinations, immediately after treatments, except in samples treated with LF showed higher ($P<0.05$) values of maximum force. After 60 d at 8 ºC, a decrease ($P<0.05$) in this parameter was observed in dry-cured ham treated with LF alone or in combination with high pressure and an increase ($P<0.05$) in shear force was detected in 450 MPa

treated samples in combination with LPS. However, no significant differences in both textural parameters were registered in treated samples compared to control dry-cured ham at the end of the investigated period.

High pressure treatments slightly increased the pH of dry-cured ham, although differences between samples tended to level off at the end of the refrigerated storage. Values of a_w increased in pressurized samples, although differences were not enough to alter food safety of product.

Treatments at 450 MPa for 5, 10 and 15 min were assessed in beef carpaccio inoculated with *S*. Enteritidis stored for 30 d at 8 ºC. After applying 450 MPa for 5 or 10 min, reductions of 3.68 and 5.94 log units were achieved. *Salmonella* was only detected after sample enrichment in samples treated at 450 MPa for 15 min. Pathogen counts only decreased 0.26 log units in non-pressurized samples after 30 d of storage at 8 ºC, indicating the ability of *Salmonella* to survive in this meat product. During the 30 d of storage at 8 ºC, *Salmonella* counts only decreased 0.26 log units in non-pressurized samples, indicating the ability of the pathogen to survive in beef carpaccio. Reductions in *Salmonella* counts were 1.33 log units with the least intense treatment and its presence in 10 g of the product was only detected after enrichment of samples treated with the most intense treatments, indicating that the pathogen were not able to recover under the assessed conditions.

An increase of TVC was detected in beef carpaccio during the 8 ºC storage period. Counts of high pressure treated samples were significantly (P<0.05) lower than those of control samples.

High pressure treatments at 450 MPa for 5, 10 and 15 min increased lightness (L^*) of beef carpaccio. This effect was observed during refrigerated storage, with an upward trend in pressurized samples at 450 MPa for 15 min. Redness values (a^*) decreased in pressurized carpaccio after treatments. The lowest values were detected at the end of the refrigerated period in samples treated at 450 MPa for 15 min. Yellowness values (b^*) were lower (P<0.05) in pressurized samples than in control samples, although differences were attenuated during storage at 8 ºC.

On one hand, shear force decreased (P<0.05) in pressurized carpaccio at 450 MPa for 5, 10 and 15 min, immediately after applying the treatment, although differences were lower with higher pressurization holding time. On the other hand, no significant differences in maximum force were detected between pressurized and control carpaccio after treatments. Both texture parameters increased during storage at 8 ºC.

With the objective to inactivate *L. monocytogenes*, *S*. Enteritidis and *E. coli* O157:H7 were inoculated on beef carpaccio and to observe the behavior of the three pathogens in beef carpaccio at temperature abuse conditions and at room temperature storage (22 º C) during 7 d, high pressure treatment at 450 MPa for 5 min, the LPS, ALF and their combinations were applied. These pathogens were not able to grow in treated carpaccio during the investigated period and moreover, the levels of *S*. Enteritidis decreased during the refrigerated storage. High pressure treatments caused a bactericidal effect against all three pathogens. *S*. Enteritidis showed the highest sensitivity to pressurization, followed by *E. coli* and *L. monocytogenes*.

No antimicrobial activity was detected in beef carpaccio when the LPS or ALF were applied. A synergistic antimicrobial effect was observed for HHP in combination with the LPS against *S*. Enteritidis and *E. coli* O157:H7. At the end of the storage, higher reductions with combined treatments of HHP and LPS were observed at 22 ºC for *L. monocytogenes* and *E. coli* O157:H7. However, the effect was more intense for *Salmonella* at 8 ºC. No enhancement of the antimicrobial potential of HHP was detected in the present work when this technology was applied in combination with ALF.

Reductions of 3.28, 3.24 and 3.55 log units were detected in TVC in carpaccio treated with the LPS, ALF or at 450 MPa for 5 min, after treatments. When high pressure and ALF or the LPS were combined, reductions of 3.69 and 3.82 log units were registered. A TVC increase was observed in non-treated and treated samples after 30 d at 8º C. TVC increase in treated samples was lower than in non-treated beef carpaccio.

When HHP was applied alone or in combination with antimicrobials, L^* values after pressurization were significantly ($P<0.05$) higher than in those control samples. A decrease ($P<0.05$) of lightness was observed in samples treated with ALF but not with the LPS. After 30 d at 8 ºC, L^* values were attenuated and no significant differences were detected among treatments, except in samples treated with ALF alone or in combination with HHP, with an increase or a decrease of this parameter, respectively.

Immediately after pressurization, the individual application of the LPS did not negatively affect redness or yellowness values of beef carpaccio. After 30 d at 8 ºC, an increase in a^* values was observed in pressurized samples and a decrease of this parameter was detected in samples treated with ALF. Regarding yellowness, a decrease in b^* ($P<0.05$) was detected at the end of the refrigerated period, in those treated with ALF and in those HHP treated in combination with the LPS.

No significant differences in shear strength values (N) were observed immediately after pressurization, except in samples treated at 450 MPa combined with the LPS, where an increase (P<0.05). After 30 d at 8 ºC, shear strength values increased (P<0.05) in samples treated with antimicrobials alone and decreased in samples where HHP were combined with the LPS. Hardness (N) determined as maximum force was not significantly affected by treatments, immediately after pressurization. After 30 d at 8 ºC, an increase (P<0.05) of this property was only detected in samples treated with LPS.

Next combination assessed in this product against *L. monocytogenes*, *S*. Enteritidis and *E. coli* O157:H7 was the application of 450 MPa for 5 min in combination with enterocins A and B, pediocin PA-1, nisin Z and commercial nisin or pediocin preparations. The application of these bacteriocins produced reductions between 0.1 and 1.5 log units in *L. monocytogenes* counts. The commercial pediocin preparation, the most effective bacteriocin against this pathogen whereas nisin Z was the less effective one. All these bacteriocins did not show antimicrobial activity against *S*. Enteritidis or *E. coli* O157:H7.

HHP at 450 MPa for 5 min was very effective against *S*. Enteritidis, with reductions higher than 4 log units, followed by *E. coli* O157:H7 and *L. monocytogenes* that showed the highest barotolerance of these 3 pathogens.

Immediately after pressurization, a slightly synergistic antimicrobial affect was detected against *Listeria* when combined treatments of enterocins A and B or commercial nisin and high pressure. However, when the combination of pediocin PA-1, nisin Z or commercial pediocin and HHP were combined, the antimicrobial effect was only additive. No enhancement of the antimicrobial potential of HHP against *S*. Enteritidis was detected in the present work when this technology was applied in combination with bacteriocins. A slightly synergistic antimicrobial effect against *E. coli* was observed with most of the combined treatments, except the combination of pediocin PA-1 that produced and additive antimicrobial effect. At the end of the storage, the antimicrobial effect against *Listeria* was additive for combinations of enterocins A and B or commercial nisin and HHP, and slightly synergistic for nisin Z or commercial pediocin and pressurization. For *Salmonella*, an additive antimicrobial effect detected when enterocins A and B or pediocin PA-1 were combined with high pressure. For *E. coli*, a synergistic antimicrobial effect was detected when enterocins A and B, pediocin PA-1 or commercial nisin were combined with HHP, with inactivation rates 2 log units higher than those achieved by adding the individual effects. During the storage at 8 ºC, these pathogens did not grow in non-treated carpaccio.

Pressurization reduced TVC in 2.14 log units and the application of enterocins A and B, pediocin PA-1 or nisin Z alone produced reductions of 0.36, 0.55 y 0.28 log units, respectively. The commercial nisin reduced TVC by 1.18 log units, being the most effective bacteriocin. The combined treatment of HHP and commercial pediocin caused a synergistic antimicrobial effect with reductions of 0.88 log units higher than the sum of each single treatment. During the storage at 8 ºC, TVC levels increased 0.94 log units in non-treated carpaccio samples. A slightly synergistic antimicrobial effect was detected in samples treated with commercial nisin or pediocin at the end of the storage. A TVC recovery was detected in the rest of samples.

The application of these bacteriocins did not affect L^* values. However, an increase of lightness was observed with all combined treatments, although this rising was only significantly higher ($P<0.05$) in samples HHP treated in combination with pediocin PA-1 or commercial nisin. After 30 d at 8 ºC, L^* values decreased ($P<0.05$) in samples treated with enterocins A and B and increased ($P<0.05$) in samples treated with commercial pediocin.

The a^* values (redness) decreased ($P<0.05$) both in HHP treated samples by themselves or in combination with bacteriocins, immediately after pressurization. Only nisin Z application decreased ($P<0.05$) a^* values. However, redness tended to attenuate for all treatments at the end of the storage. After pressurization, b^* (yellowness) tended to increase in samples where HHP was applied alone or combined with bacteriocins, although this decrease was only significant ($P<0.05$) when pediocin PA-1 or commercial pediocin were combined with HHP. After 30 d at 8 ºC, differences in b^* values tended to attenuate. Results obtained in the present work show that the changes detected in beef carpaccio were mainly due to the effect of HHP and the time of storage and not to bacteriocins.

No significant differences were observed in shear strength values (N), immediately after pressurization. After 30 d at 8 ºC, this property increased ($P<0.05$) in samples treated with enterocins A and B, pediocin PA-1, nisin Z, or commercial pediocin, and also in pressurized samples combined with nisin Z or commercial nisin.

Hardness significantly increased ($P<0.05$) in samples treated with commercial pediocin alone or in combination with high pressure, immediately after pressurization. At the end of the storage, this parameter increased ($P<0.05$) in non-treated samples and in those treated with enterocins A and B alone or combined with high pressure.

High pressure treatments slightly increased pH values in beef carpaccio and the application of pediocin PA-1, comercial nisin or 450 MPa for 5 min combined with comercial nisin produced a decrease in pH, immediately after pressurization. After 30 d at 8 ºC, pH values decreased in non-treated samples. The a_w values increased in high pressure treated carpaccio alone or in combination with the LPS or ALF. An increase in a_w values was also detected in carpaccio treated with enterocins A and B, pediocin PA-1, nisin Z or combinations of 450 MPa for 5 min and enterocins A and B or commercial nisin.

The effect of combined treatments of sodium nitrite and high pressure on the inactivation of *E. coli* and *L. monocytogenes* has only been investigated in buffer, but bearing in mind their possible application in meat products. Where indicated, sodium nitrite was added to the buffer at a concentration of 0.25, 0.50, 0.75, 1.0, 2.0 y 3.0 mM. Native cells of *E. coli* and *L. monocytogenes* were sensitive to sodium nitrite at pH 4.0. Exposure of *E. coli* to 1.0, 2.0 and 3.0 mM acidified sodium nitrite caused an immediate reduction in viable counts of <1 log but after storage at 4 ºC for 24 h log reductions increased to 1.10, 2.60 and 5 logs, respectively. Regarding *L. monocytogenes*, the presence of 0.25, 0.50 and 0.75 mM acidified sodium nitrite caused reductions of 0.90, 2.30 and 3.60 log units, respectively, after 24 h at 4 ºC.

When *E. coli* was exposed to a pressure of 300 MPa for 8 min, reductions of 1.40 log units were achieved. If this pressure treatment was combined with 1.0 or 2.0 mM sodium nitrite, viable levels decreased by 2.4 and 4.90 log units, respectively, whilst with 3.0 mM nitrite viable counts were below detection limits (<2.4 log cfu/mL), indicating reductions of >6 logs. The degree of inactivation 24 h later was higher than 6 logs in all the combined treatments. With *L. monocytogenes*, a pressure treatment at 225 MPa for 8 min caused about a 1 log reduction in viability. When combined with 0.25, 0.50 or 0.75 mM sodium nitrite, decreases of 1.50, 2.70 and 4.20 log units, respectively, were obtained. An increase (P<0.001) in inactivation was observed 24 h later achieving reductions of 3.4, 6.10 and 6.10 log units, respectively.

The combined treatment of sodium nitrite and high pressure was synergistic against *E. coli* and *L. monocytogenes*. The effect of pH on high pressure combined with 2.0 mM nitrite acidified at pH 4.0 was studied. Treatment at pH 4.0 caused reductions of about 4 logs immediately after treatment, but no effect was recorded at pH 5.0 or pH 7.0. However, a slightly synergistic antimicrobial effect was observed at pH 4.5 when 3.0 mM nitrite was used. Under these conditions, the combined treatment caused a reduction of 1.40 log compared with 0.23 log for the sum of separate treatments.

The pH dependence is consistent with the bactericidal effect of nitric oxide derived from nitrous acid.

With the objective to study the combined effect in the detail, *E. coli* BW25113 wild type and *E. coli* BW25113 *hmp*A and *nor*V mutants, lacking the flavohaemoglobin nitric oxide dioxygenase and nitric oxide reductase, respectively (Gardner *et al*., 2002; Poole, 2005) were used. The mutants were more sensitive to acidified nitrite than the parent strain and even more sensitive to the HHP treatment alone. Moreover, differences in synergistic antimicrobial effects between strains were not statistically significant.

CONCLUSIONS

High hydrostatic pressure at 500 or 600 MPa for 5 min were effective on the inactivation of *S*. Enteritidis in sliced dry-cured ham. A recovery of the pathogen was not detected during the storage of the product in temperature abuse conditions.

The combined treatment of 400 or 500 MPa for 10 min and commercial nisin showed a synergistic antimicrobial effect against *E. coli* O157:H7 in sliced dry-cured ham. This effect was kept during the storage of the product when 500 MPa and commercial nisin were combined.

The combination of 450 MPa for 10 min and the lactoperoxidase system or lactoferrin caused a synergistic antimicrobial effect against *S*. Enteritidis in sliced dry-cured ham. The antimicrobial effect achieved with the combination of 450 MPa and the lactoperoxidase system was synergistic at the end of the refrigerated period against *L. monocytogenes*.

Changes on texture and color of dry-cured ham were slight. Pressurization caused a slight increase of lightness and lower redness, as well as some modifications of texture. Such changes tended to attenuate during storage.

Treatments at 450 MPa for 5, 10 and 15 min were effective on the inactivation of *S*. Enteritidis in refrigerated beef carpaccio. A reduction of 5 log units in *Salmonella* counts was achieved with the least intense treatment.

The combined treatments at 450 MPa for 5 min and the lactoperoxidase system or activated lactoferrin were effective on the inactivation of *S*. Enteritidis and *E. coli* O157:H7 in beef carpaccio. The combination of 450 MPa and the lactoperoxidase system showed a synergistic antimicrobial effect against *S*. Enteritidis and *E. coli* O157:H7.

Pressurization of beef carpaccio at 450 MPa for 5 min or the combination of this high pressure treatment with enterocins A and B, pediocin PA-1, nisin Z, commercial nisin or pediocin were effective to inactivate *S*. Enteritidis and *E. coli* O157:H7. In case of *E. coli* O157:H7, a synergistic antimicrobial effect was detected when the high pressure treatment and enterocins A and B, pediocin PA-1 or commercial nisin were combined.

Treatments modified slightly color and texture of beef carpaccio, with a slight increase of lightness and lower redness. Hardness increased at the end of the refrigerated period with the most intense treatments.

A bactericidal effect of sodium nitrite applied alone or in combination with high pressure was observed against *E. coli* and *L. monocytogenes in vitro* studies. The combined antimicrobial effect was synergistic at pH 4.0 against both microorganisms.

The *E. coli* BW25113 *hmp*A and *nor*V mutants were more sensitive to acidified nitrite and high pressure than *E. coli* BW25113 wild type pointing at the involvement of nitric oxide in the bactericidal effect.

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